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JOURNAL OF LIPID RESEARCH

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Volume 1
Number 1

Editorial

Development of a Journal

The JOURNAL OF LIPID RESEARCH, like many other periodicals, is the result of growing necessity. For a number of years workers in the lipid field have felt an increasing need for better exchange of information, particularly in lipid methodology. Because of the limited space available for publication of the many new procedures of lipid analysis, it could easily be foreseen that communication would be the rate-limiting factor in lipid research.

The original proposal was to prepare a loose-leaf handbook of methodology which would be kept current by yearly supplements. It was even suggested that prior to their acceptance for inclusion in the handbook, the methods be tested by one or more laboratories.

Although in theory these ideas were good, their application did not appear practicable. It would take too long to state in detail the reasons for proceeding with the publication of a scholarly journal; however, the decision was made only after careful consideration of all other possibilities. The majority reaction has been that the advantages of this endeavor far outweigh all possible disadvantages, and it is hoped that the new journal will be viewed as a healthy sign of the growth of biochemical research in general and the upsurge of lipid investigation in particular.

The JOURNAL OF LIPID RESEARCH, a quarterly, will offer to its readers a representative selection of original work in the chemistry, biochemistry, enzymology, histochemistry, and physiology of the lipids. In addition, the editors will, at regular intervals, invite reviewers to summarize and discuss significant advances in their specialized fields. One section, "Notes on Methodology," will be devoted to the prompt publication of modifications of existing lipid methods. Another section, "New Methods," a comprehensive listing of new lipid methods currently published in other scientific journals, will be prepared by the editorial staff. Those concerned with the publication of the JOURNAL will depend heavily upon the advice of experienced, mature scientists, whose constructive criticisms will always be gratefully received.

The editors have made every effort to keep the control of the JOURNAL in the hands of research workers themselves. To accomplish this purpose, a nonprofit organization, Lipid Research, Incorporated, was formed by the charter members: Drs. Edward H. Ahrens, Jr., Joseph H. Bragdon, Herbert E. Carter, Jules Hirsch, Irvine Page, Henry Sebrell, and Donald B. Zilversmit. A grant from the National Heart Institute made it possible for this group to obtain the editorial and business experience of University Publishers Incorporated, another nonprofit organization, in New York City. The financial support of the JOURNAL OF LIPID RESEARCH is thus derived entirely from the Public Health Service grant and subscriptions received from individuals and institutions.

Another feature of the new journal worth mentioning is its international scope. We have been extremely fortunate in obtaining the co-operation of a most distinguished group of scientists from abroad. As corresponding editors they will keep the JOURNAL and its readers informed of the advances in lipid research outside the United States. It is hoped that a great many of the articles published in the JOURNAL will be contributed by our colleagues in other countries.

It is fully expected that the JOURNAL will be of value to investigators concerned with basic lipid research and that it will give the lipid field the recognition it deserves.

D. B. ZILVERSMIT
Editor

University of Tennessee
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J. Lipid Research
October, 1959

Editorial

On Certain Conventions

A word of explanation concerning the spelling of the name of this JOURNAL seems in order, inasmuch as a few journals have for some years insisted that the proper spelling of the word is "lipide." The decision was not lightly made. Considerable research and discussion preceded the final choice. Anyone interested in a historical account of the origin of the terminal *e* is referred to a column by Dr. A. M. Patterson in *Chemical and Engineering News* 30: 1910, 1952. The principal argument in favor of the *e* was that of maintaining consistency with the spelling of such chemical terms as sulfide, oxide, glyceride, amide, and so forth. We feel that this argument is really irrelevant, as the word lipid does not stand for a specific chemical structure. Arguments in favor of the shorter form include the fact that the second *i* is almost always pronounced short, not long, as it should be with a terminal *e*. It is also a fact that most journals, both in this country and abroad, use "lipid."

Another choice, and one far more arbitrary, faced the editors in selecting a name, and an abbreviation, for those fatty acids in the blood which are not bound in covalent linkage and which have come much into vogue in recent years as "unesterified fatty acids" (UFA), "nonesterified fatty acids" (NEFA), "free fatty acids" (FFA), and even "albumin-bound fatty acids" (ABFA). Reasons for avoiding "unesterified fatty acids" and "nonesterified fatty acids" include the fact that the fatty acids of sphingomyelin also are not in ester linkage. In addition, UFA can be confused with "unsaturated fatty acid," and NEFA with "nonessential fatty acid." "Albumin-bound fatty acids" is not strictly correct as some of these fatty acids are apparently bound to lipoprotein. "Free fatty acids" was finally adopted in full knowledge of the fact that they are not "free" in the sense of being nonprotein bound (although a very small percentage is—depending on the dissociation constants of the complexes formed by the individual acids and proteins), but they are free in the sense of "not bound by covalent linkages." Similar usage exists, for example, in the term "free cholesterol." Furthermore, "free fatty acids" corresponds with the chemical concept of "titratable acidity readily extractable from a slightly acid medium by a nonpolar solvent." Finally one school of thought holds that abbreviations should not be pronounceable. Certainly no one can pronounce FFA.

J. H. BRAGDON
Associate Editor

National Heart Institute
Bethesda 14, Maryland

Volume 1
Number 1

Review

Biosynthesis of fatty acids and cholesterol considered as chemical processes

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National Institute for Medical Research, London, N.W. 7, England

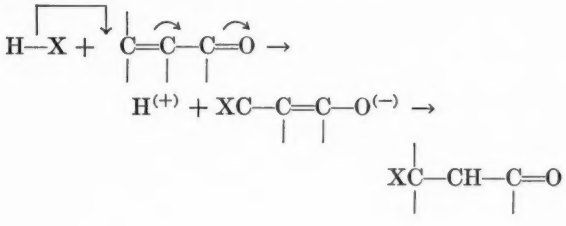
[Received for publication June 25, 1959]

The expansion of knowledge concerning biosynthesis of lipids has become almost explosive; and any review of its present state is likely to be overlaid by new developments by the time it is printed. Enough is already known, however, to show that lipids, in comparison with proteins and carbohydrates, are built up by an unusually wide variety of enzymic reactions representing many different chemical types. This article attempts a survey, from the viewpoint of an organic chemist, of the processes leading to biosynthesis of fatty acids and of cholesterol. It might be objected that enzymic transformation of a substrate can rarely be matched—for speed and adaptability to a cool substantially neutral aqueous medium—by the same transformation executed *in vitro* without enzymes; and therefore that procedures which are possible or impossible in a chemical laboratory have no necessary relation to the capabilities of a living cell. Yet few enzymic reactions are known which have no counterpart in organic chemistry: considered as chemical transformations of substrate into product, most are of quite familiar type. If enzymes, like other components of living matter, have undergone evolution and adaptation, they could be expected to mediate processes which are not too difficult to effect without specific catalysis. The apo-enzyme would have undergone progressive development of its power specifically to adsorb reactants and to facilitate the charge-transfers, in substrate and coenzyme, necessary for reaction. The entropy and energy of activation are thereby progressively lowered; but if they were initially too high, evolution could not have progressed. The function of an enzyme being so to provide an ideally favorable environment for an inherently facile chemical change, the ordinary laws of chemical reactivity can be used to discuss and predict the course of biosynthesis. The justification of this approach is found in its many successful predictions and in its power to limit the number of hypotheses to be tested by experiment; its dangers lie in the temptation to accept a chemically plausible mechanism without critical testing, and in the tendency to regard as a single chemical step an enzymic process which may prove to contain several.

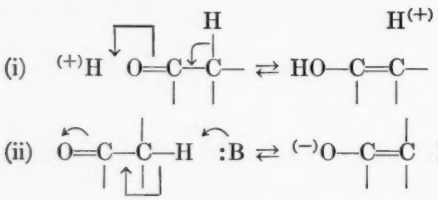
BIOSYNTHESIS OF FATTY ACIDS

The biosynthesis of fatty acids appears to be controlled almost entirely by one source of chemical reactivity: the permanent polarization of a carbonyl group, $\rangle C{=}O$. This polarization arises from the fact that although carbon and oxygen atoms are of the same size and have identical orbitals available to their electrons, oxygen has a higher nuclear charge than carbon and therefore tends to hold in its vicinity more than a half share in the electrons of a bond linking the two atoms. Thus the carbon atom has a fractional positive charge, and from this electron-deficiency comes its reactivity to nucleophilic reagents.

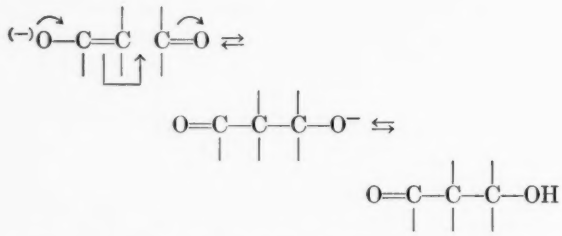
When a C=C double bond is conjugated with a carbonyl group, as in —CH=CH—C=O, the β-carbon also becomes electron-deficient and can add a nucleophilic reagent, generalized here as HX:



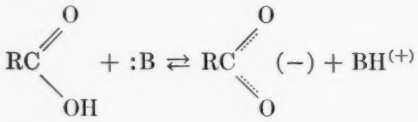
A further consequence of the polarization of a carbonyl group is that formation of an enol or its anion can easily be induced by (i) acidic or (ii) basic reagents, a hydrogen atom on the α-carbon being removed:



Both the enol and the enolate ion are *nucleophilic* reagents, and it is this easy transformation of an electrophilic carbonyl group to a nucleophilic enol which makes the group so important a generator of new carbon-carbon bonds. Enolization is a reversible reaction; but instead of recombining with the lost α-hydrogen atom, an enol or its anion may combine with other electron-deficient atoms, including a nonenolized carbonyl group:

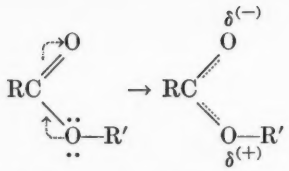


In carboxylic acids and their derivatives (esters, amides, anhydrides, and the like) the carbonyl group is attached to a heteroatom which can modify its reactivity profoundly. In a carboxylic acid, a base readily removes a proton to give the stable symmetrical anion in which, because of the distributed negative charge, the electron-deficiency at carbon is very slight.



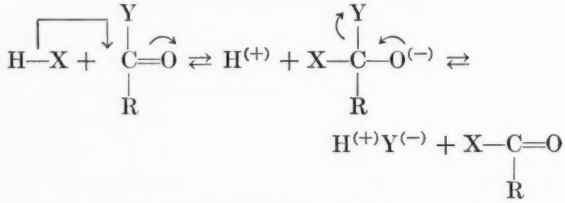
Thus bases add to or enolize the carboxyl group with great difficulty, and even when the undissociated form of the acid can react (as in acid-catalyzed esterification) a deactivation similar to that of esters (see below) makes addition more difficult.

In esters, RCOOR′, unshared electrons of the singly-bound oxygen atom tend to take part in the C—O bond, making it a partial double bond and largely neutralizing the positive charge on carbon:



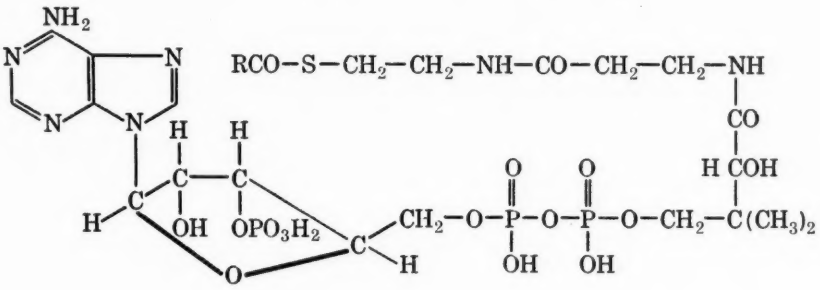
This effect is strong enough to overcome the tendency, already mentioned, for oxygen to attract electrostatically the electrons of a carbon-oxygen bond, and it is even stronger in amides RCONR′R″, in which the carbonyl group is consequently even more unreactive.

A third class of carboxylic acid derivatives can be grouped as anhydrides. When the acyl group, RCO—, is attached to the anion of an acid HY, the group Y influences reactions at the carbonyl group in two related ways. Since HY is an acid (i.e., it tends to dissociate into $H^{(+)}Y^{(-)}$), the group Y necessarily is electron-attracting, and in the compound RCOY it withdraws electrons from the carbonyl carbon, accentuating its electron-deficiency. Thus, unless a deactivating effect like that shown by esters can occur, the electrophilic reactivity of the carbonyl group is actually greater than in an aldehyde or ketone. Further, when addition of a nucleophilic agent to the carbonyl group has occurred, elimination of the stable $Y^{(-)}$ anion can take place so readily that the replacement of Y by X may be virtually irreversible.

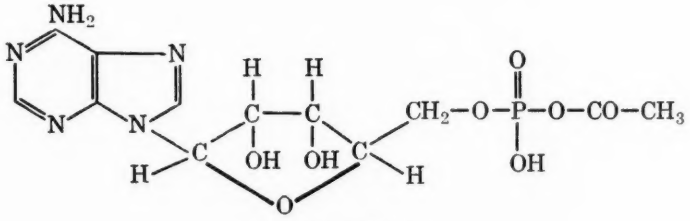


In an acid chloride, RCOCl, the electron-attracting power of the chlorine atom is little reduced by the slight tendency of its unshared electrons to participate in the C—Cl bond, and acid chlorides are among the most reactive of carbonyl compounds. In carboxylic acid anhydrides, RCOOCOR′, the deactivating effect seen in esters is lessened, since the same oxygen atom must supply two carbonyl groups. To acyl phosphates, $RCOOPO_3H_2$, similar considerations apply. Finally, in thiolesters, RCOSR′, the sulfur atom is electron-attracting and its unshared electrons have less tendency, relative to oxygen, to participate in the C—S bond. The carbonyl group in thiolesters is not so reactive as in acid chlorides or carboxylic acid anhydrides, but decidedly more reactive than in esters, amides, or acids.

A chemist looking for a carboxylic acid derivative which, while being stable enough to persist in neutral aqueous solution, should undergo enolization and nucleophilic attack as readily as possible, could hardly find a better compromise than a thiolester offers, though perhaps he would consider the *p*-nitrophenyl esters (1) which have proved serviceable for peptide synthesis. The structure of coenzyme A thiolesters (I) appears complex: no doubt much of the molecule is adapted to specific adsorption on an enzyme; but it is the thiolester link which conveys the necessary reactivity to the acyl group COR.



(I)



Acetyl adenylate (II)

Activation of Fatty Acids. Enzymic formation of coenzyme A thiolesters of fatty acids by direct combination of acid with coenzyme A is energetically unfavorable, the free energy change on esterification being (2) of the order of +8000 cal./Mol. Thiolester formation must therefore be coupled with a reaction yielding energy. Two systems for activation of acetate have been recognized. The first, mediated by the enzyme acetate-thiokinase, is the more widespread (3 to 7), occurring in animal tissues as well as in green plants and microorganisms; and here the necessary energy is supplied by the cleavage of adenosine triphosphate to adenylic acid and pyrophosphate.

$$CH_3CO_2H + CoASH + ATP \rightleftarrows CH_3COSCoA + AMP + PP$$

The equilibrium constant of this balanced reaction is of the order of unity (5); Lynen and Decker (8) pointed out that pyrophosphatase is associated with acetate-thiokinase in practically all cells, and probably serves to displace the equilibrium in the direction of thiolester synthesis.

The experimental evidence, due principally to Berg (9), concerning the mechanism of this change may be summarized thus: (i) the reversible change is effected by a single enzyme; (ii) the only auxiliary factor known to be required is magnesium ion; (iii) synthetic acetyl adenylate (II) can replace adenosine triphosphate plus acetate, and its addition to a complete system sharply reduces consumption of adenosine triphosphate; (iv) acetyl adenylate could not be shown to accumulate in the absence of coenzyme A; (v) exchange of C^{14}-acetate and acetyl-coenzyme A by the enzyme requires adenylic acid, pyrophosphate, and magnesium ions; (vi) exchange of C^{14}-adenylate with adenosine triphosphate requires acetate and coenzyme A; (vii) adenyl acetate is converted into adenosine triphosphate by the enzyme in the presence of pyrophosphate and magnesium ion; (viii) exchange of P^{32}-pyrophosphate with adenosine triphosphate by the enzyme requires acetate and magnesium ion but not coenzyme A; (ix) acethydroxamic acid is formed enzymically from adenosine triphosphate, magnesium ion, acetate, and hydroxylamine in

the absence of coenzyme A; (x) magnesium is not required for the formation of acetyl-coenzyme A from acetyl adenylate, coenzyme A, and the enzyme.

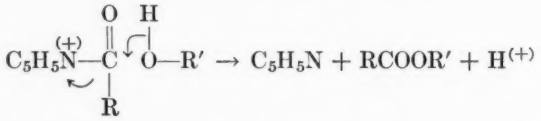
Except for the fact that its accumulation could not be induced, this is strong evidence that acetyl adenylate is an intermediate. This failure led Ingraham and Green (10) to formulate the change as a gathering of reactants within a single enzyme-magnesium complex in such a way that displacement reactions between them are favored by reduction of the entropy of activation: not acetyl adenylate, but its nondissociating magnesium chelate, is the intermediate. Berg (11) pointed out, however, that this view does not readily accommodate finding (x) quoted above.

To prepare a thiolester of acetic acid from the acid and the thiol, a chemist would first make from the acid an anhydride or mixed anhydride, and treat this with the thiol. His procedure seems analogous to the sequence induced by acetate-thiokinase; but having no enzymes, he might facilitate the second stage by use of a tertiary amine. A base such as pyridine greatly accelerates the acylation of an alcohol or phenol by an acyl chloride or anhydride; it does this by forming a saltlike complex in which the acyl cation is bound to the pyridine nitrogen and the chloride ion is free:

$$C_5H_5N + RCOCl \rightleftarrows C_5H_5\overset{(+)}{N}\text{—}COR\ Cl^{(-)}$$

The reaction is reversible, and it is interesting that stabilization of similar acyl cations can be achieved by adding a complexing agent (antimony pentachloride) to remove chloride ion (12).

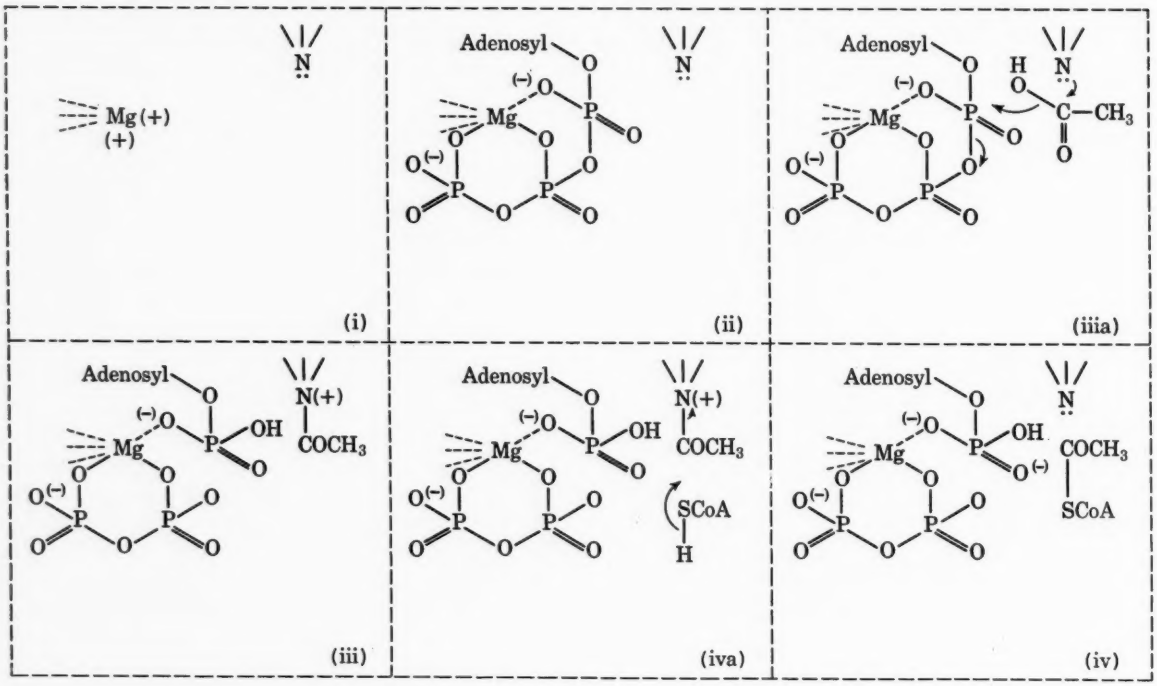
The cation is highly reactive to nucleophilic reagents (12, 13), e.g., it will form esters rapidly with alcohols, and anhydrides with acids (14):



$$C_5H_5\overset{(+)}{N}\text{—}\underset{\underset{R}{|}}{\overset{\overset{O}{\|}}{C}}\text{—}O\text{—}R' \rightarrow C_5H_5N + RCOOR' + H^{(+)}$$

By postulating that the change mediated by acetate-thiokinase may be base-catalyzed, a structure for an active center on the enzyme can be formulated; the mechanism is in some respects a reconciliation of the ideas of Berg and of Ingraham and Green. The active center would comprise a basic group (e.g., a tertiary amine) surrounded by receptors for (*a*) a magnesium ion; (*b*) adenylate; (*c*) acetate; (*d*) coenzyme A. It is assumed that (*b*) and (*c*) can act in concert as a receptor of acetyl adenylate, and (*c*) and (*d*) as a receptor of S-acetyl-coenzyme A; (*a*), occupied by magnesium, is a receptor for pyrophosphate or, in concert with (*b*), of adenosine triphosphate.

The sequence of events in the forward reaction



SCHEME 1

would then be (i) attachment of magnesium; (ii) attachment of adenosine triphosphate; (iii) attack by an acetate ion or acetic acid molecule to give pyrophosphate and a dissociated base-complex of acetyl adenylate; (iv) attack of a coenzyme A molecule to give S-acetyl-coenzyme A; (v) dissociation of products from the enzyme. All these stages, taken in their proper order, are assumed to be reversible. The state of the enzyme on completion of stages (i) to (iv) is illustrated (Scheme 1) as well as—in (iii*a*) and (iv*a*)—the electron-shifts of stages (iii) and (iv).

Acetyl adenylate is not an intermediate in this mechanism: when synthetic acetyl adenylate is attached to the enzyme, it passes to an ionized state, as in (iii). The acetyl-adenylate bond has high energy and its dissociation by the basic group could well be independent of the participation of magnesium.

A possible experimental test might be made of the dissociation of acetyl adenylate on the enzyme. Berg (9) found that ^{14}C-acetate did not exchange with acetyl adenylate when both were incubated on the enzyme, but if in the absence of magnesium the adenylate ion were not firmly bound, exchange of adenylate with acetyl ^{14}C-adenylate might be demonstrable.

A second activating system, found so far only in bacteria, consists of two enzymes, acetokinase (15) and phosphotransacetylase (16), which respectively catalyze the reversible changes:

$$\text{Acetate} + \text{ATP} \rightleftarrows \text{Acetyl phosphate} + \text{ADP}$$

$$\text{Acetyl phosphate} + \text{CoASH} \rightleftarrows \text{Acetyl—SCoA} + \text{phosphate}$$

Acetokinase requires magnesium or manganous ion; phosphotransacetylase does not; the two enzymes may in fact present, separately, functional sites similar to those found combined in acetic-thiokinase.

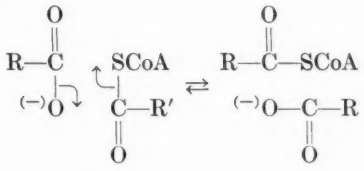
The thiophorases (CoA-transferases) effect activation of carboxylic acids by exchange with a molecule of an S-acyl-coenzyme A. Thus acyl-acetate-thiophorase from *Clostridium kluyveri* (17) is a relatively unspecific enzyme effecting the interchange:

$$\text{Acetyl—SCoA} + \text{carboxylic acid anion} \rightleftarrows \text{acyl—SCoA} + \text{acetate}$$

and succinyl-acetoacetate-thiophorase from mammalian heart muscle and kidney (18, 19) is important for the activation of acetoacetate:

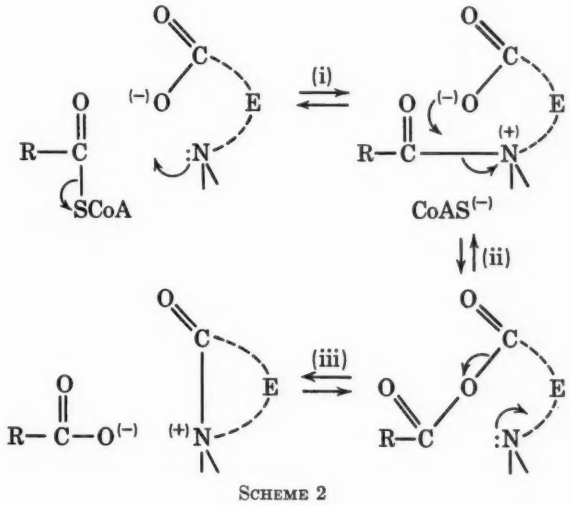
$$\text{Succinyl—SCoA} + \text{acetoacetate} \rightleftarrows \text{acetoacetyl—SCoA} + \text{succinate}$$

The free energy change in these reversible processes is naturally not large; but the mechanism, as affecting the energy of activation, is of some interest. The simplest mechanism which can be written is a cyclic one, e.g.,



Though this possibility cannot be dismissed, the four-membered cyclic transition state is unusual for single bonds. A tracer experiment (20) bearing on the question is the reported exchange of ^{14}C-succinate with S-succinyl coenzyme A on the enzyme. This is not compatible with direct exchange unless succinate can occupy the site adapted for acetoacetate. Ochoa (21) therefore suggested that the primary reaction is with a carboxyl group of the enzyme to form an "S-enzymyl" coenzyme A which then transfers coenzyme A to the receptor acid, the reaction thus being composed of two separate but similar exchanges of undetermined mechanism.

From the chemical standpoint it seems most likely that the anhydridelike character of an S-acyl coenzyme A permits a base-catalyzed equilibrium with a carboxylic anhydride, which in turn undergoes base-catalyzed reaction with coenzyme A. If the enzyme (E) contained a basic group and a carboxylate ion in suitable spatial relationship, a possible sequence would be as in Scheme 2.



SCHEME 2

The products of stage (iii) are the carboxylate anion of the acid originally esterified by coenzyme A, and an "activated enzyme," an acyl cation which could give a new S-acyl coenzyme A by attachment of a new carboxylate anion ($R'\text{-}COO^{(-)}$) and reversal of all three stages. Experimental testing of this and other possibilities would seem to be not too difficult, in view of the highly purified preparations available.

Formation of Carbon-Carbon Bonds. Until recently it was thought that synthesis and breakdown of coenzyme A thiolesters of fatty acids proceed by repetitions of a single sequence of reversible changes, the carbon chain being lengthened or shortened by two units at the completion of each sequence:

$$\text{RCOSCoA} + \text{CH}_3\text{COSCoA} \underset{\beta\text{-keto thiolase}}{\overset{(1)}{\rightleftharpoons}} \text{CoASH} + \text{RCOCH}_2\text{COSCoA}$$

$$\text{RCOCH}_2\text{COSCoA} \overset{(2)\ \beta\text{-hydroxybutyryl dehydrogenase}}{\rightleftharpoons} \text{RCHOHCH}_2\text{COSCoA}$$

$$\text{RCH=CHCOSCoA} \overset{(3)\ \text{enoyl hydrase}}{\rightleftharpoons} \text{RCHOHCH}_2\text{COSCoA}$$

$$\text{RCH=CHCOSCoA} \overset{(4)\ \text{acyl dehydrogenase}}{\rightleftharpoons} \text{RCH}_2\text{CH}_2\text{COSCoA}$$

Most of the available evidence concerns the simplest case (R = CH_3), where all four reactions in both directions have been demonstrated with purified enzymes. For the higher fatty acids, evidence for the degradative sequence remains good, but evidence for step (1) of the synthetic sequence is lacking.

Step (1), chemically, is an acetoacetic ester condensation. This reversible reaction, when brought about between two ester molecules, requires a base which can enolize one molecule, the enolate ion then attacking the carbonyl group of the other. Thus the two molecules of S-acetyl coenzyme A which participate in the formation of S-acetoacetyl coenzyme A have necessarily different roles, and their environment on the enzyme must also be different: one site facilitating ionization of the α-hydrogen and the second the separation and reattachment of a thiol group. It has been suggested (22) that actual formation of an S-acetyl enzyme occurs at this second site:

$$\text{CH}_3\text{COSCoA} + \text{ESH} \rightleftarrows \text{CH}_3\text{COSE} + \text{CoASH}$$

$$\text{CH}_3\text{COSE} + \text{CH}_3\text{COSCoA} \rightleftarrows \text{CH}_3\text{COCH}_2\text{COSCoA} + \text{ESH}$$

Two pieces of evidence (22) support this view: (i) the enzyme, acetoacetyl-thiolase, is inhibited by iodoacetate, arsenoxide, etc.; (ii) S-propionyl coenzyme A exchanges rapidly with S^{35}-coenzyme A in presence of the enzyme. There is, however, no evidence about the actual location of the supposed sulfhydryl group in the enzyme, and in any case the site which necessarily facilitates separation and reattachment of coenzyme A in the enzymic reaction could be expected also to facilitate exchange. It was formerly thought that the asymmetric labeling observed in acetoacetate produced enzymically from 1-C^{14}-fatty acids or from C^{14}-acetyl coenzyme A was additional evidence for an intermediate S-acetyl enzyme, but the data seem to be explained more simply as an operation of the "hydroxymethylglutarate cycle" (23). The condensation with a second molecule of S-acetyl coenzyme A remains, of course, of the same type whether it is with S-acetyl coenzyme A or S-acetyl enzyme.

In the presence of acetoacetyl-thiolase, the cleavage of S-acetoacetyl coenzyme A is much faster than its synthesis, the equilibrium constant (19, 24, 8)

$$\frac{[\text{Acetoacetyl—SCoA}][\text{CoASH}]}{[\text{Acetyl—SCoA}]^2}$$

lying between 10^{-4} and 10^{-5} at physiological pH. In thiolesters of higher fatty acids, the carbonyl group is certainly less reactive to nucleophilic reagents (e.g., the S-acetyl coenzyme A enolate) than it is in an acetyl thiolester, and this is probably why the thiolase-catalyzed synthesis of a long-chain S-β-oxoacyl coenzyme A from S-acetyl coenzyme A has not been observed, the higher energy of activation making the energy barrier, already steep, almost impassable.

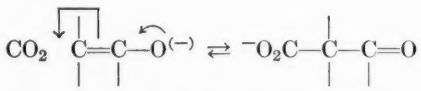
An important advance in understanding the biosynthesis of the higher fatty acids was the recent recognition of carbon dioxide as an essential cofactor. Gibson, Titchener, and Wakil (25, 26) obtained from avian liver a highly purified soluble enzyme system which synthesized long-chain fatty acids (chiefly palmitate) from S-acetyl coenzyme A in the presence of adenosine triphosphate, manganous ion, reduced triphosphopyridine nucleotide, and bicarbonate ion. Little synthesis of higher fatty acids occurred without carbon dioxide, but carbon dioxide was not a source of carbon for the product. Examination of the system failed to show the presence, in significant amount, of various enzymes mediating the breakdown of fatty acids.

Brady (27) prepared the mono-coenzyme A thiolester $HO_2C\text{—}CH_2\text{—}COSCoA$, by enzymic activation of malonate and showed that in the presence of acetaldehyde, manganous ions, and a soluble fraction

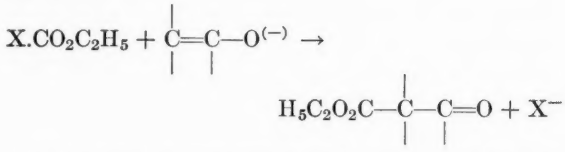
from pigeon liver this gave other coenzyme A thiolesters with mobility on paper chromatograms corresponding to chain-lengths of 8 to 12 carbon atoms. Wakil (28) and Formica and Brady (28*a*) later reported that S-malonyl coenzyme A could be obtained enzymically from S-acetyl coenzyme A in the presence of adenosine triphosphate, manganous or magnesium ion, and bicarbonate.

Recently Wakil and Ganguly (29) reported on two fractions from avian liver. The first of these contained biotin and synthesized S-malonyl coenzyme A from S-acetyl coenzyme A and bicarbonate in the presence of adenosine triphosphate and manganous ions; the second with S-malonyl coenzyme A gave intermediates which passed, on addition of reduced triphosphopyridine nucleotide, to carbon dioxide and fatty acids.

At the time of writing, evidence concerning the role of S-malonyl coenzyme A is still fragmentary, and a discussion of the chemical reactivities of the substance seems profitable; its formation from carbon dioxide and S-acetyl coenzyme A should be examined first. This reaction is of familiar type, the carboxylation of an enol:

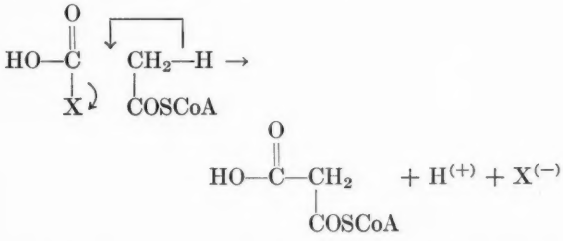


The oldest example is the classic synthesis (30) of salicylic acid from sodium phenoxide and carbon dioxide, but many others are known. Alternatively, the enolate ion can react with an appropriate derivative of carbonic acid, e.g., ethyl carbonate or ethyl chloroformate.

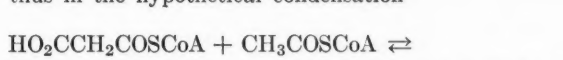


The course of carboxylations *in vitro* suggests that the principal energy barrier to be overcome is in the ionization of the carbonyl compound, e.g., a ketone enolized by reaction with a high-energy reagent such as sodium triphenylmethide can be carboxylated by pouring it over solid carbon dioxide (31).

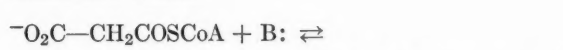
In the enzymic carboxylation of S-acetyl coenzyme A the adenosine triphosphate is presumably concerned with conversion of carbon dioxide to an intermediate of higher energy (HO.CO.X), which may then attack the methyl carbon of S-acetyl coenzyme A as the hydrogen atom is withdrawn, fission of C—H and C—X bonds being simultaneous:



S-Malonyl coenzyme A could *prima facie* be utilized in several ways for the biosynthesis of fatty acids. Extension of the carbon chain might be effected by condensation with (*a*) an S-acyl coenzyme A, or (*b*) an aldehyde. To discuss these possibilities it is necessary to consider how S-malonyl coenzyme A differs from an ordinary S-acyl coenzyme A in its reactivities. The electrophilic reactivity of the thiolester group would be that of an ordinary S-acyl coenzyme A: thus in the hypothetical condensation

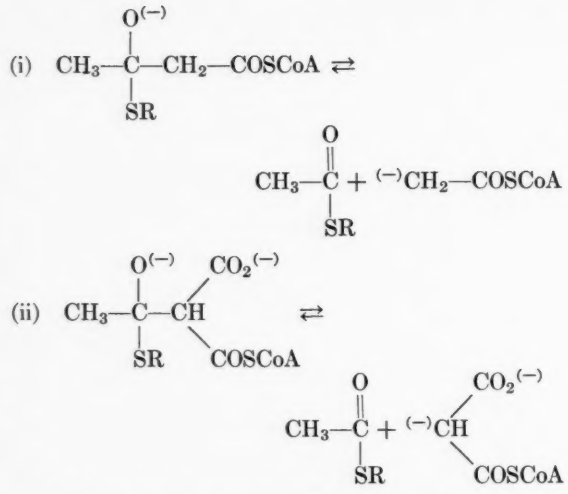


S-malonyl coenzyme A would have no advantage. The principal effect of the additional carboxyl group is to facilitate the enolization of the ester: the enolate ion would be formed with much greater ease than, for example, from S-acetyl coenzyme A, because the negative charge of the ion is distributed over a larger mesomeric system.

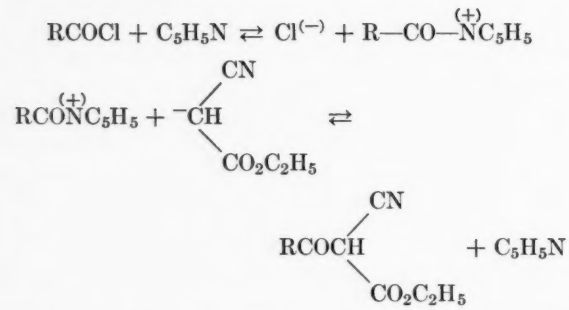


Neutralization of the additional negative charge on the carboxylate group, e.g., by complexing with a cation, would further stabilize the enolate.

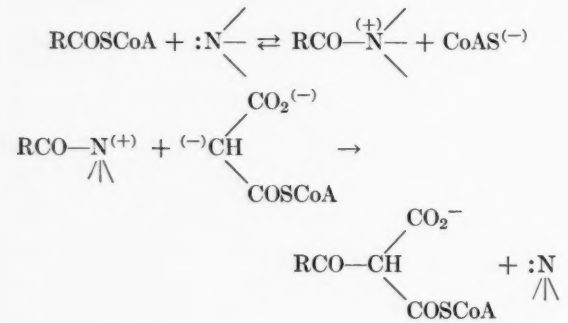
The condensation of this enolate with an S-acyl coenzyme A by an enzyme of the thiolase type does not seem likely to lead to a greater degree of synthesis than occurs in the condensation of two molecules of S-acetyl coenzyme A. Indeed, when the two equilibria (i) and (ii) are compared, the second should lie farther to the right because of the greater stability of the enolate ion; and in fact acylmalonic esters cannot be made *in vitro* by the usual acetoacetic ester condensation. It has been necessary, when making acylmalonic esters and their derivatives, to bring together an enolate of the malonic ester with an acyl derivative of the anhydride type, usually an acyl chloride. It is



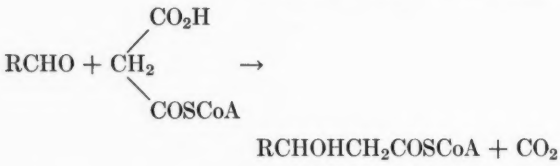
particularly interesting that C-acylation of a malonic acid derivative, e.g., ethyl cyanoacetate (32), can be effected by reaction in the cold with an acyl chloride and a tertiary base (pyridine or quinoline). The intermediate here would be an acylammonium cation of a type already discussed.



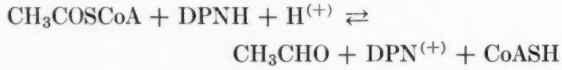
Thus it seems possible that the enzyme effecting condensation of an S-acyl coenzyme A with S-malonyl coenzyme A has a basic center which effects dissociation of the acyl derivative to a cation which then reacts with the malonyl enolate:



A different scheme, proposed by Brady (27), assumes reduction of an S-acyl coenzyme A to an aldehyde, which then undergoes condensation with S-malonyl coenzyme A.



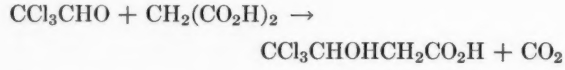
A system reducing S-acetyl coenzyme A in the presence of reduced triphosphopyridine nucleotide was present in Brady's fatty acid synthesizing system. Burton and Stadtman (33) found an enzyme in *Clostridium kluyveri* mediating the change:



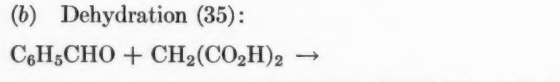
The reduction to acetaldehyde was endergonic to the extent of 4330 cal. per mol; with triphosphopyridine nucleotide as the reducing agent this figure would be only slightly lower.

Condensation of an aldehyde with a malonic acid derivative is easy to effect nonenzymically with relatively mild reagents, and the reaction has often been used preparatively; however, the initial condensation is often followed by dehydration or decarboxylation or both. Indeed, no example is known to the writer of the isolation of the simple addition product, a β-hydroxymalonic acid, without dehydration or decarboxylation, provided that the malonic acid derivative had at least one free carboxyl group. Some examples of known reaction types are:

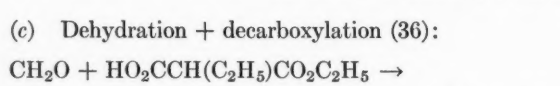
(*a*) Decarboxylation (34):



(*b*) Dehydration (35):



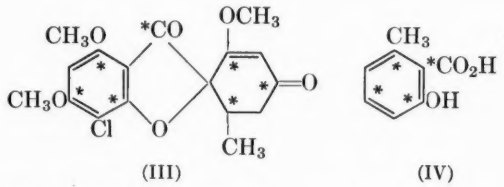
(*c*) Dehydration + decarboxylation (36):



If Brady's hypothesis is correct, the reaction which actually extends the carbon chain is facilitated by two energy-requiring steps: reduction of a thiolester to an aldehyde and carboxylation of the other reagent, S-acetyl coenzyme A. The stage at which the superfluous carboxyl group would be eliminated remains uncertain, but its continued presence would certainly facilitate reduction of the double bond formed by (also facile) dehydration. Chemically, the condensation is well supported by analogy; however, the actual utilization of a fatty aldehyde as such in the biosynthesis of a higher fatty acid has yet to be demonstrated.

All these schemes utilized, for extension of a carbon chain, the electrophilic nature of the thiolester group at the end of the existing chain; this is so whether direct formation of a C—C link occurs or whether reduction to an aldehyde comes first. Theoretically, it is not indispensable for this thiolester group to be attached to a saturated alkyl residue (methyl, propyl, etc.); the thiolester group in S-β-hydroxybutyryl coenzyme A, for example, should be as reactive as that in S-butyryl coenzyme A. In S-crotonyl coenzyme A, $CH_3CH{=}CHCOSCoA$, the electrophilic activity of the carbonyl group is decidedly lowered by the double bond; but in S-vinylacetyl coenzyme A, $CH_2{=}CHCH_2COSCoA$, there is no conjugation and the carbonyl group would be fully reactive. This is also true of S-malonyl coenzyme A, $HO_2CCH_2COSCoA$, and even of S-acetoacetyl coenzyme A, $CH_3COCH_2COSCoA$, if the more reactive keto group is prevented in some way from enolizing or otherwise reacting preferentially.

In recent years Birch (37, 38) has demonstrated that a considerable number of aromatic substances, most of them produced by fungi, are derived from acetic acid units arranged in head-to-tail sequence. In such compounds an oxygen atom is frequently found attached to a carbon originating from acetate carboxyl: a good example is griseofulvin (III). The labeling pattern in 6-methylsalicylic acid (IV) biosynthesized from 1-^{14}C-octanoic acid by *Penicillium griseofulvum* indicates dissociation of the fatty acid into one or more C_2 units before synthesis. No study has yet been made

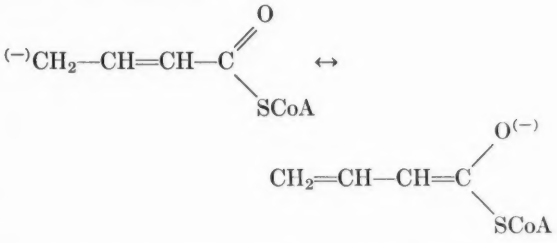


(III) (IV)

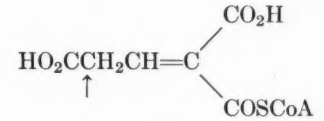
* Signifies carbon originating from acetate carboxyl.

of the enzymes effecting these biosyntheses, and it is still unknown whether coenzyme A plays its part in them, but it is at least possible that the processes are similar to fatty acid syntheses, the removal of oxygen sometimes being omitted before the next C_2 unit is added.

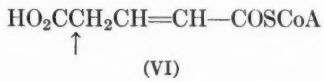
A further possibility for the building up of the carbon chain in fatty acids is the addition of four carbon atoms en bloc. S-Crotonyl coenzyme A could in theory form an enolate ion,



in which nucleophilic activity is transferred to C—4 via the double bonds. Lapworth (39) was able to condense ethyl crotonate with ethyl oxalate to give the straight-chain keto ester, $C_2H_5O_2CCOCH_2CH{=}CHCO_2C_2H_5$; a reaction in which a similar enolate ion must take part. Self-condensation of S-malonyl coenzyme A as part of the process of fatty acid synthesis could lead to intermediates (V and VI) from which more stable enolate ions could be formed, by loss of hydrogen at the arrowed positions, for reaction with a thiolester or an aldehyde; the intermediate (VI) could also arise by carboxylation of S-crotonyl coenzyme A.



(V)



(VI)

The only experiments known to the writer which suggest that four-carbon addition may occur are those of Hele *et al.* (40), where a cell-free system utilizing acetate synthesized $C_{(4)}$ and $C_{(8)}$ acids rapidly, with $C_{(6)}$ only appearing at a later stage.

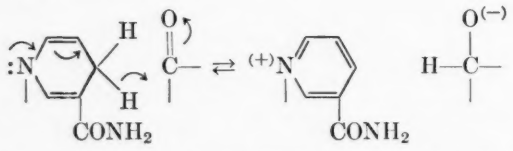
Hydrogen-transferring Processes. When fatty acids are built up from S-acetyl coenzyme A, two reductions necessarily occur for each $C_{(2)}$ unit added to the chain. In each of these reductions, two electrons are transferred; correspondingly, two oxidations are required

for each shortening of a fatty acid by two carbon atoms.

The reversible process whereby S-acetoacetyl coenzyme A is reduced to S-β-hydroxybutyryl coenzyme A in the presence of reduced diphosphopyridine nucleotide is representative of the first reducing step:

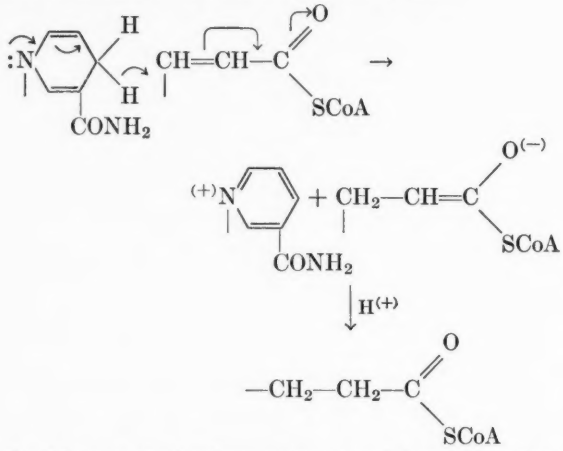
$$CH_3COCH_2COSCoA + DPNH + H^+ \rightleftarrows CH_3CHOHCH_2COSCoA + DPN^+$$

In general, reduction of a keto group to a secondary alcohol is easily effected nonenzymically by a considerable range of reducing agents. Enzymic reductions of this type are not confined to lipid biochemistry; and in this case (41), as with alcohol dehydrogenase (41*a*) and lactic dehydrogenase (42), it has been shown that reduction and oxidation are a direct transfer of "negative" hydrogen between the pyridine nucleotide and the substrate.



It should be pointed out that the various schemes for fatty acid synthesis via S-malonyl coenzyme A, discussed in the previous section, do not eliminate the need for a reduction of this type. The reduction of S-acyl coenzyme A to aldehyde required by Brady's hypothesis is a reduction of essentially the same kind. Certainly, the nonenzymic reduction of esters to aldehydes is difficult, but the thiolester group in S-acyl coenzyme A would facilitate nucleophilic attack by hydrogen. Also, attachment of the group —COSR to the enzyme in a particular configuration could facilitate reduction; a similar effect is seen in *cis*-lactones (43), which have a fixed configuration of the ester group and have greater electrophilic activity than esters. The reduction of gluconolactone to glucose (44) by so mild a reagent as sodium amalgam is a familiar example.

The second reducing step in fatty acid synthesis is presumably the reduction of an $\alpha\beta$-unsaturated coenzyme A ester, possibly carboxylated at the α-position. Formerly it was thought that reduction by one of the flavoprotein acyl dehydrogenases was on the principal pathway of synthesis, but this now appears doubtful, since these enzymes are absent from the synthesizing system of Gibson *et al.* (26). This system utilizes reduced triphosphopyridine nucleotide, as does also another (apparently different) system, found in extracts of liver, which reduces $\alpha\beta$-unsaturated coenzyme A esters (45, 46, 47). A reduction of this type can be regarded as similar to that of a ketone, but attack by the negative hydrogen would be on the β-carbon made electrophilic by transmission of the C=O polarization through the conjugated system.



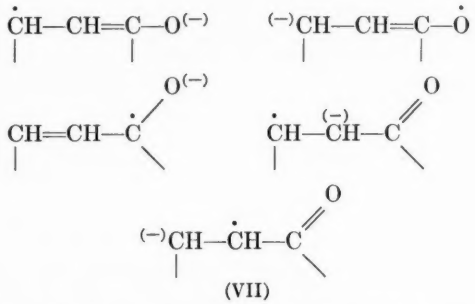
In this way the hydrogen transferred from the reducing agent would appear on the β-carbon of the saturated ester.

Chemical reduction of the C=C double bond in an $\alpha\beta$-unsaturated acid or ester can be brought about rather easily, e.g., by alkali metals and alcohols; and if $\alpha\beta$-unsaturated ketones are also considered (since coenzyme A thiolesters approach them in reactivity) the range of possible reducing agents is wider. Of the reverse process, dehydrogenation of a fatty ester or ketone to its $\alpha\beta$-unsaturated analogue, no example is known. Thus the reaction catalyzed by acyl-dehydrogenases is of considerable chemical interest; a discussion is therefore included despite the doubt concerning its role in biosynthesis of fatty acids. It has always been found difficult to drive this reaction in the direction of hydrogenation.

The mechanism of the dehydrogenation has been studied chiefly by Beinert and collaborators (48). Spectroscopic evidence indicates that an intermediate stage is produced by transfer of one electron from substrate to flavin. Beinert and Page's reasonable explanation of the considerable body of experimental fact is that the intermediate is in the nature of a bi-radical complex ($\cdot$YH.....HS$\cdot$) of partially reduced enzyme and partially oxidized substrate (enzyme and substrate are respectively Y and SH_2). This intermediate is relatively stable; the second electron-transfer needed to complete dehydrogenation requires an auxiliary factor, the "electron-transferring flavoprotein" (ETF).

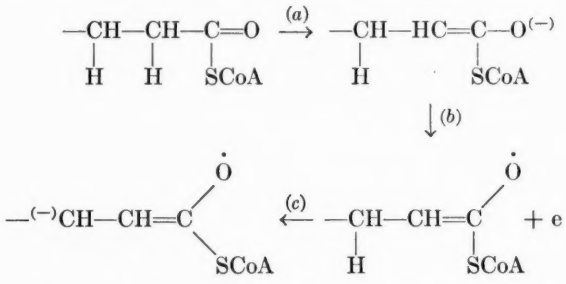
This reasoning can perhaps be given a more precise

formulation by considering the structure of the entity called (·SH). The relatively long life of this radical, even as a complex with the semiquinonoid flavin radical, argues a certain stability. Among possible half-reduction products of an $\alpha\beta$-unsaturated carbonyl compound the radical-ion (VII) produced by simple addition of one electron appears to be the most stable, since at least five canonical structures are possible between which resonance should occur.

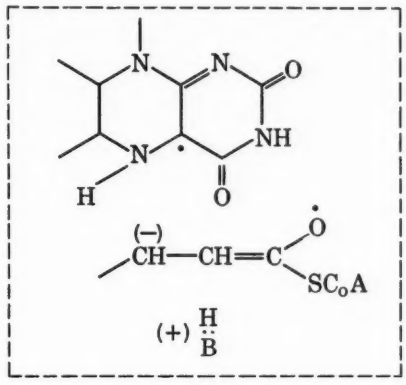


(VII)

On this view the radical derived from SH_2 is not ·SH but ·$S^{(-)}$. Its formation by oxidation of a saturated S-acyl coenzyme A can be formulated as follows:

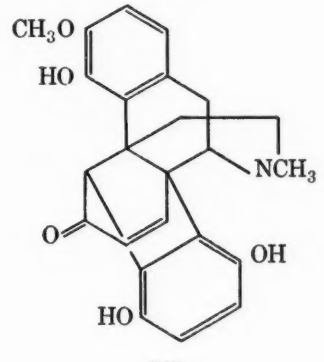


Stage (*a*) is a base-catalyzed enolization of the thiolester; in stage (*b*) the enolate ion donates one electron to the flavin, losing its negative charge and giving a free radical which passes in (*c*) to the stabilized radical-ion by loss of a proton from the β-carbon. The separation of this proton would be facilitated by a strong base, and it is interesting that the transfer of an electron in stage (*b*) would produce in the flavin a strongly basic radical-ion, which could accept the proton. The three stages can in fact be amalgamated as a continuous cyclic process: as abstraction of an α-hydrogen in the substrate by a basic center in the enzyme begins, the electronic excess so generated is transferred to the flavin, raising its basicity so that it can assist in abstraction of the β-hydrogen. The structure of the bi-radical complex would then be as (VIII). When an $\alpha\beta$-unsaturated thiolester reacts with the reduced enzyme (YH_2), removal of a proton from the reduced flavin is followed by transfer of one electron from flavin to substrate to produce the same intermediate.



(VIII)

The nature of the electron-transfer is of particular interest; most probably, it would be the result of orbital overlap between the two mesomeric systems in flavin and substrate. This overlap would persist in the complex and help to stabilize it. It is not easy to find a known chemical model, but a parallel can perhaps be seen in the alkaloid derivative flavothebaone (49, 50). In this substance (IX) a hydroquinone ring and an $\alpha\beta$-unsaturated keto group, though not in conjugation, are fixed at a separation of about 3 Å. Flavothebaone is yellow and its alkaline solution is deep red, though both should be colorless if no interaction occurred between the two chromophores. Electronic interaction between the two components of the bi-radical (VIII) might thus occur over a distance greater than the length of an ordinary covalent bond. The transfer of a second electron after oxidation or reduction of the flavin radical in the complex (VIII) would lead to the fully reduced or oxidized product.



(IX)

Enoyl Hydrase (*Crotonase*). This enzyme, which has been crystallized (51), catalyzes the reversible addition of water to $\alpha\beta$-unsaturated S-acyl coenzyme A. The $\beta\gamma$-unsaturated analogues are also hydrated, but proof is lacking that the process is reversible. As with the acyl-dehydrogenases, the part played by crotonase in the synthesis, as opposed to the degradation, of fatty acids, is at present uncertain. However, some type of dehydration of an S-β-hydroxyacyl coenzyme A or its α-carboxylated analogue would seem to be essential to most of the possible schemes involving S-malonyl coenzyme A.

With crotonase, the known relationships in the four-carbon series can be summarized (52, 53):

$$CH_3CH{=}CHCOSCoA \rightleftarrows L(+)\text{-}CH_3CHOHCH_2COSCoA$$

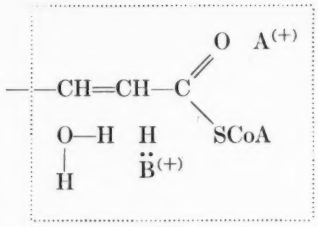
$$\uparrow$$

$$CH_2{=}CH{-}CH_2COSCoA$$

The reversible hydration of $\alpha\beta$-unsaturated carbonyl compounds is a well-known process in organic chemistry; with $\alpha\beta$-unsaturated ketones it may occur under very mild conditions. That this intrinsic facility includes coenzyme A thiolesters is shown by Rendina and Coon's observation (54) that S-acrylyl coenzyme A, $CH_2{=}CH{-}COSCoA$, and S-methacrylyl coenzyme A, $CH_2{=}C(CH_3){-}COSCoA$, are hydrated spontaneously in aqueous solution. With $\alpha\beta$-unsaturated ketones the most effective catalysts for hydration are acids, and for dehydration bases. The electron-shifts would be as shown in Scheme 3.

No comparable mechanisms exist for the $\beta\gamma$-unsaturated isomers.

Crotonase could thus be pictured as having an acidic group (A^+) near the carbonyl group of the attached substrate, a basic group (B:) near an α-hydrogen atom, and possibly a group such as a metallic cation near the β-carbon atom for addition of water molecules and abstraction of hydroxyl ions. (Stern and del Campillo suggested (52) that a sulfhydryl group adds to the β-carbon and is later exchanged for hydroxyl; but a chemical parallel for the second stage is hard to find.) In view of the fact that both *cis*- and *trans*-isomers of S-crotonyl coenzyme A and S-2-hexenoyl coenzyme A can be hydrated, and that crotonase will accept coenzyme A thiolesters having a wide range of chain-lengths (52), it would seem that carbon atoms beyond the β-carbon are not specifically bound to the enzyme. The structure of the enzyme-substrate complex could then be pictured as (X).



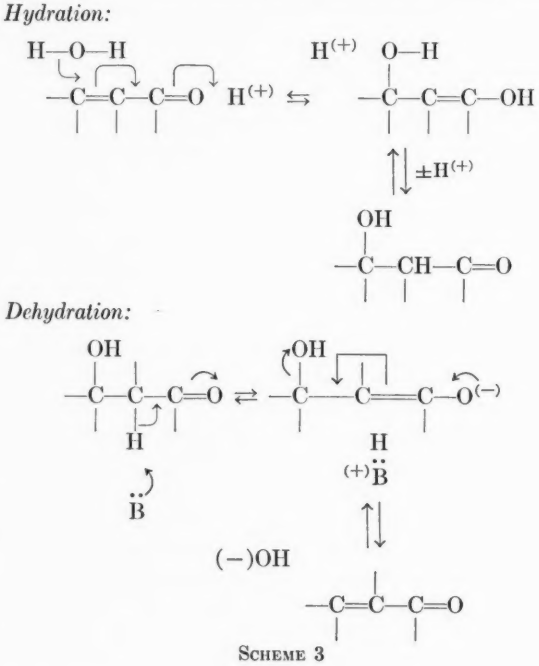
(X)

The sequences for hydration and dehydration then proceed as in Scheme 3. On this view, hydration of S-vinylacetyl coenzyme A is not direct but is preceded by isomerization to S-crotonyl coenzyme A (Scheme 4).

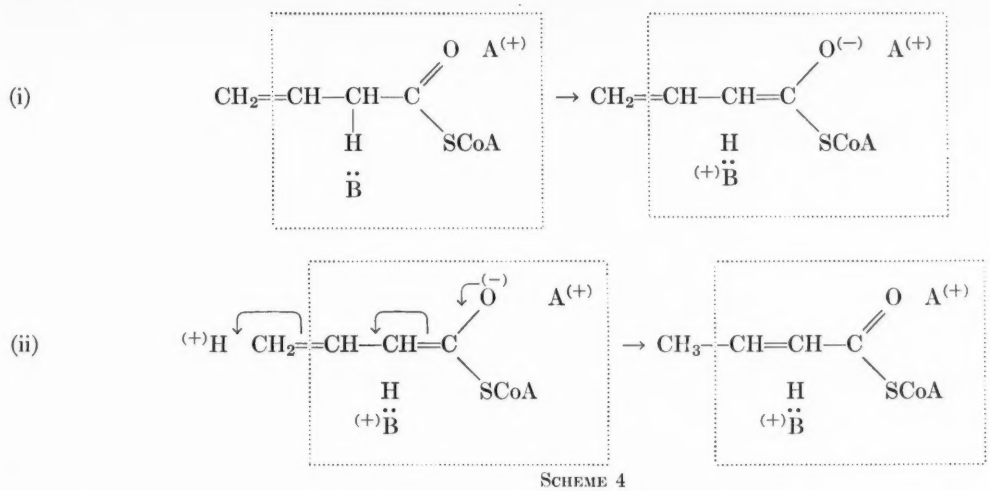
Whether the change (ii) is reversible in the absence of a site on the enzyme for removal of a proton from the γ-carbon may be doubted, and the absence of such a site would also hinder the formation of S-vinylacetyl coenzyme A from S-β-hydroxybutyryl coenzyme A. More light, it seems, could be thrown on the nature of crotonase by determining whether S-vinylacetyl coenzyme A is actually in equilibrium, in presence of the enzyme, with the $\alpha\beta$-unsaturated and β-hydroxy thiolesters.

BIOSYNTHESIS OF CHOLESTEROL

The individual enzymes mediating the biosynthesis of cholesterol have not yet received intensive study;



SCHEME 3



SCHEME 4

none, if we except acetoacetyl thiolase, has been obtained pure, and most of the evidence has been secured with rather complex preparations of living tissue. The principal purpose of this section is to relate to organic chemistry as a whole the very interesting and varied changes proceeding during biosynthesis. The chain of transformations leading from acetate to cholesterol is thought, on the basis of evidence now available, to be as shown in Scheme 5 (pp. 16-17).

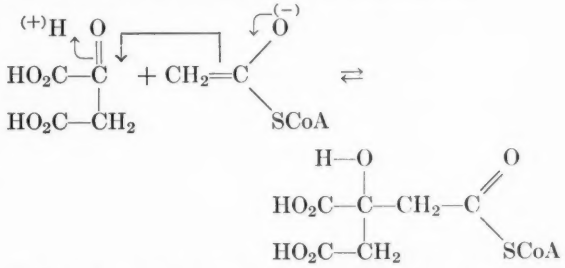
The successive discoveries of acetate (55), squalene (56), lanosterol (57), and mevalonate (58) as precursors of cholesterol provided four signposts on the way; the paths between them are being mapped out in rapidly increasing detail.

Acetate to Mevalonate. The actual transformation of acetate to mevalonate in one operation has only recently been demonstrated (59) in a preparation from rat liver of microsomes and soluble enzymes supplemented by cofactors (CoASH, ATP, Mg^{++}, GSH, TPNH). However, processes which almost certainly represent successive stages in this transformation had already been studied. The activation of acetate by acetic-thiokinase and the reversible condensation of two molecules of S-acetyl coenzyme A to give S-acetoacetyl coenzyme A can be accepted as reactions common to other systems.

The next step is the condensation of S-acetoacetyl coenzyme A with S-acetyl coenzyme A to give one molecule each of coenzyme A and S-3-hydroxy-3-methylglutaryl coenzyme A. The enzyme mediating this change ("HMG-CoA condensing enzyme") has been partially purified from yeast (23, 60); and its presence, apparently in a more labile form, in liver microsomes is also indicated.

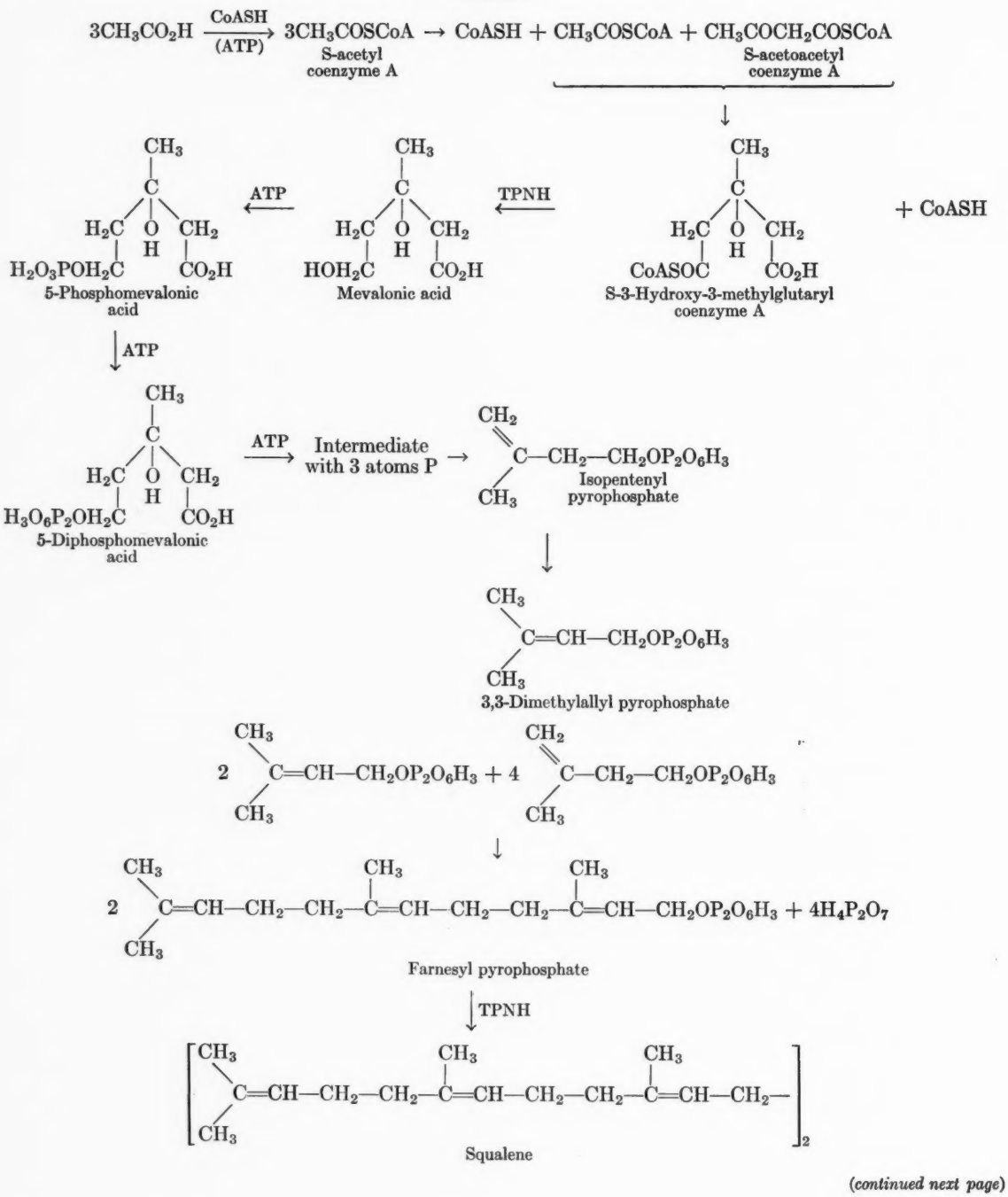
The chemical change effected by this enzyme is formally of the same type as the well-known synthesis of citrate from oxaloacetate and S-acetyl coenzyme A by the crystalline citrate condensing enzyme (61, 62); and since this enzyme has been purified and extensively studied, its mechanism can be discussed here as a close parallel. There are two interesting features of this change: the liberation of coenzyme A accompanying condensation, and the reversibility of the process (though the equilibrium lies heavily on the side of synthesis, $K \approx 10^4$ at pH 7.2).

Superficially, it would appear that the thiolester group executes its known role of facilitating the enolization of the acetyl group, the enol or enolate ion then attacking the carbonyl group of oxaloacetate:



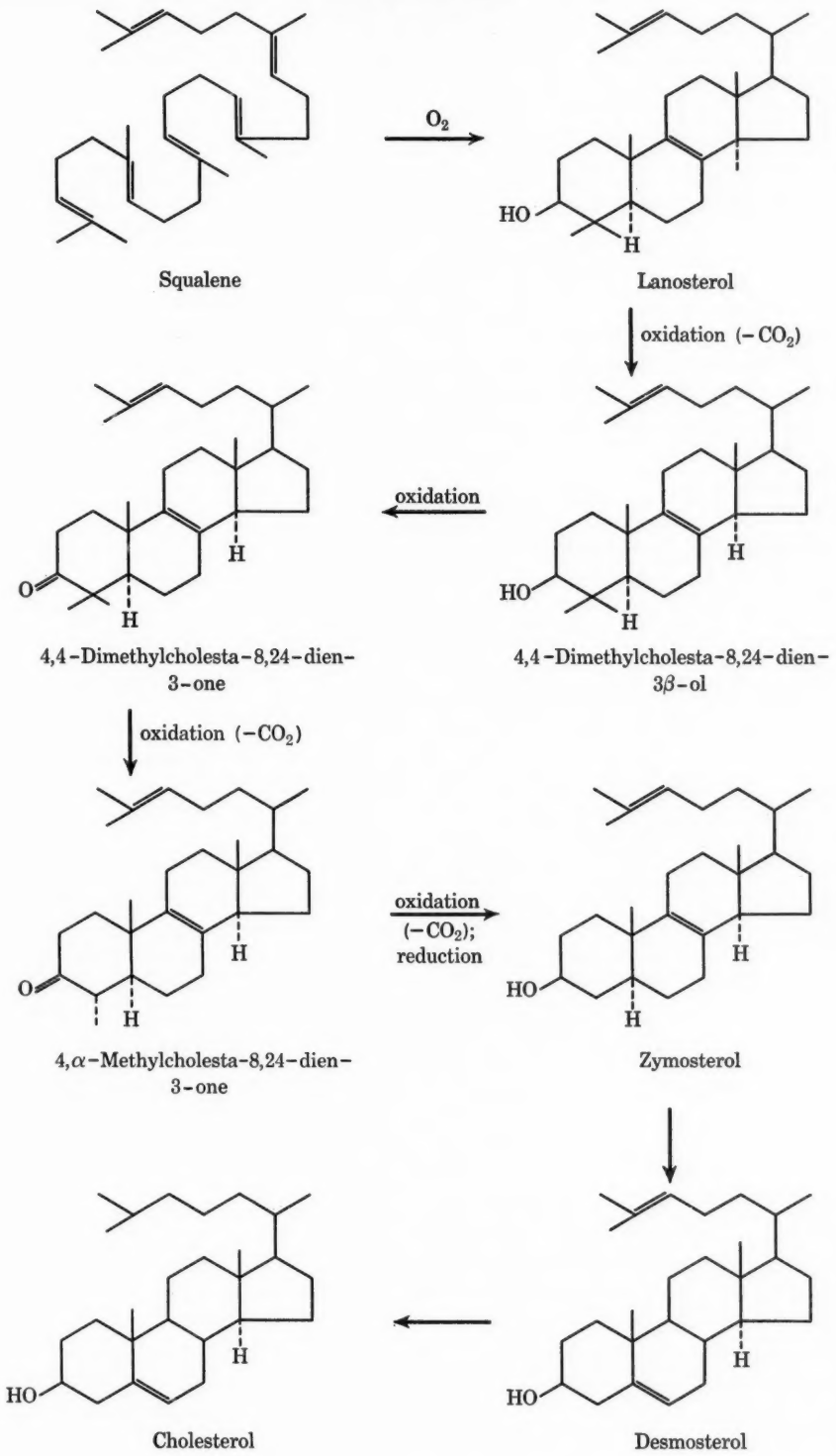
This leads to an S-citryl coenzyme A which would have to be hydrolyzed, reversibly, in a second step. There are two objections to this idea. First, S-acetyl coenzyme A does not exchange hydrogen with the aqueous medium in the presence of the enzyme and the absence of oxaloacetate (62*a*). This argues against enolization as a first step. The second objection is even more weighty: reversal of the hydrolysis would amount to the formation of a coenzyme A thiolester from a car-

SCHEME 5



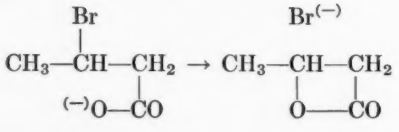
(*continued next page*)

SCHEME 5 (*continued*)

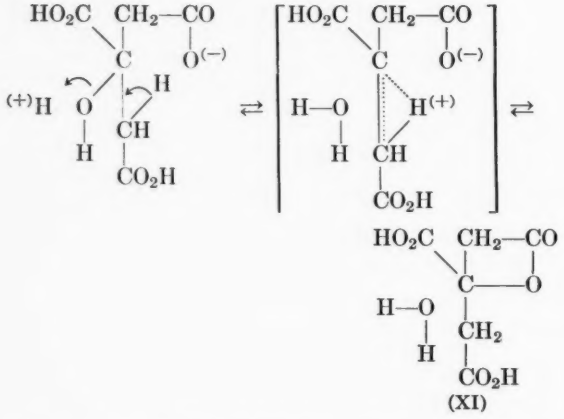


boxylic acid and coenzyme A without an external source of energy. The energy required to form the CO—S bond is so high that such a process appears most unlikely in the absence of some special characteristic of the acid concerned. In fact, S-acyl coenzyme A deacylases appear not to catalyze resynthesis; a recent example (63) is S-palmityl coenzyme A deacylase.

A possible explanation is that citric acid can be converted on the enzyme into the β-lactone (XI). Ordinary lactones are formed, nonenzymically, from hydroxy-acids by attack of the alcoholic hydroxyl group on the carbonyl group of the acid, but formation of β-lactones in this manner has never been observed. However, if an electron-deficiency can be produced on the β-carbon the carboxylate ion can attack it: thus sodium 3-bromobutyrate produces a β-lactone in neutral aqueous solution (64):



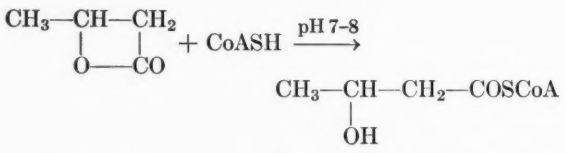
An electron-deficiency on the central carbon of citric acid could be produced by a process corresponding to the initial stage of a dehydration to aconitic acid, and the β-lactone could then be formed, thus:



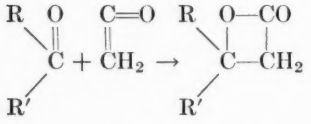
(XI)

It is a peculiarity of β-lactones that in addition to the usual reaction with nucleophilic reagents by attack on the carbonyl group, they can also be attacked at the β-carbon atom; indeed, in the hydrolysis of β-butyrolactone by molecular water this is the predominant mode (65). Thus the over-all reaction pictured above is reversible, though naturally the equilibrium would lie heavily on the side of citrate.

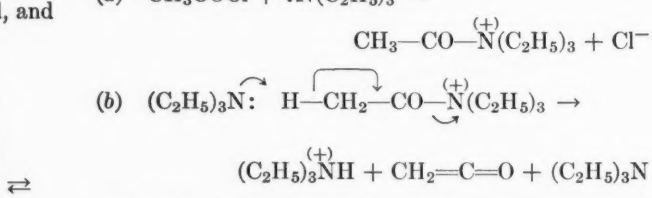
β-Lactones will, as indicated, also react with nucleophilic reagents at the carbonyl group, and indeed reaction of β-butyrolactone with coenzyme A in this sense has been demonstrated (8) *in vitro:*



The next question is whether the β-lactone (XI) could be formed from oxaloacetate and S-acetyl coenzyme A. β-Lactones can, in fact, be formed with very mild catalysis from ketones and keten (66):



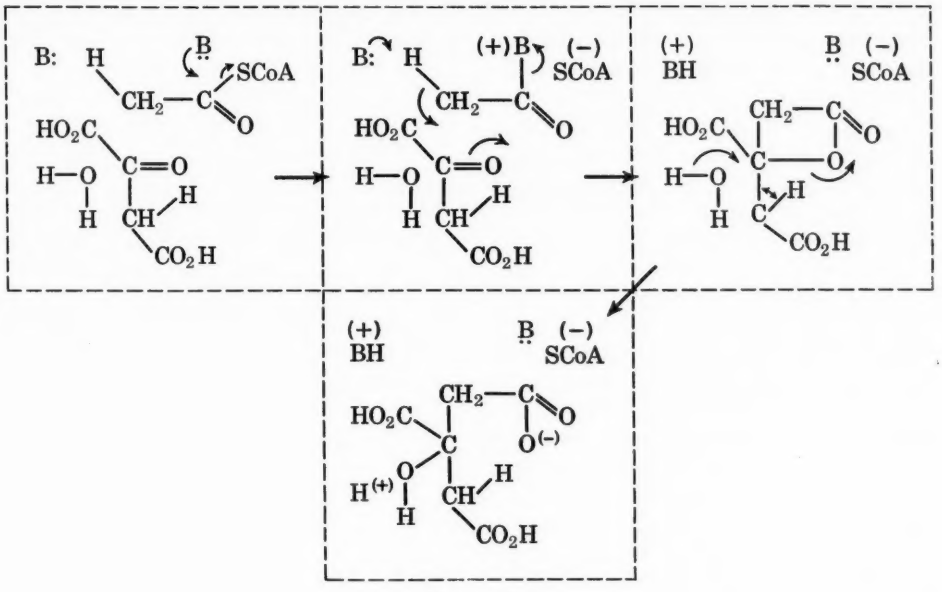
Keten is, of course, a high-energy compound, usually produced by pyrolytic methods; but it is also apparently formed from acetyl chloride and triethylamine (67), though the dimer is the product actually isolated. The mechanism of the process is presumably



two molecules of base taking part. Free keten could hardly be formed enzymically from S-acetyl coenzyme A, but it does seem possible that two basic centers on the enzyme could induce similar electron-shifts and facilitate a direct transformation of oxaloacetate to the β-lactone. These considerations lead to the following imaginary picture of the enzyme and the synthesis thereon of a molecule of citrate (Scheme 6).

Since in this mechanism little change of position is required in the atoms concerned, the entire movement of electrons may be a concerted process, the stages not being sharply divisible. In the reverse process, the electron-shifts are in the opposite direction. If this mechanism is substantially correct, the oxygen atom of the keto group in oxaloacetate becomes part of one of the carboxyl groups in citrate; a prediction which may be amenable to experimental test.

Similar mechanistic arguments apply to the condensation of S-acetyl coenzyme A with S-acetoacetyl coenzyme A to give S-3-hydroxy-3-methylglutaryl coen-

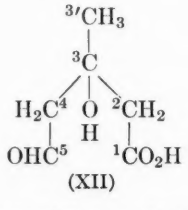


SCHEME 6

zyme A, a process which is, however, not known at present to be reversible. An enzyme (68) cleaving the product certainly exists, but it is not identical with the condensing enzyme and its products are free acetoacetate and S-acetyl coenzyme A.

Reduction of S-3-hydroxy-3-methylglutaryl coenzyme A to mevalonic acid has been reported by Lynen *et al.* (69) and by Durr *et al.* (70). The enzyme preparations were derived from yeast. Reduced triphosphopyridine nucleotide was required.

Reduction of an S-acyl coenzyme A by reduced pyridine nucleotides has been mentioned earlier (p. 10); in these examples an aldehyde was the product. The further reduction of an aldehyde to a primary alcohol is unremarkable chemically; but here both groups reported that the aldehyde concerned, mevaldic acid (XII), could not be detected as an intermediate. Mevaldic acid is, however, reduced to mevalonic acid by



(XII)

reduced triphosphopyridine nucleotide and yeast enzymes (71). A system from pig liver also effecting this reduction utilizes di- or tri-phosphopyridine nucleotide (72).

Another remarkable feature of the reduction of S-3-hydroxy-3-methylglutaryl coenzyme A to mevalonic acid is its relative irreversibility. Attempts to reverse the reduction by removing the product with the "HMG-CoA cleavage enzyme" have been reported (71), but the total conversion was very small. One would not expect this oxidation, an energy-yielding process, to be so difficult; and it is clear that this step, which seems as it were a one-way street connecting biosynthesis of cholesterol with general metabolism, will prove of unusual interest when studied in detail.

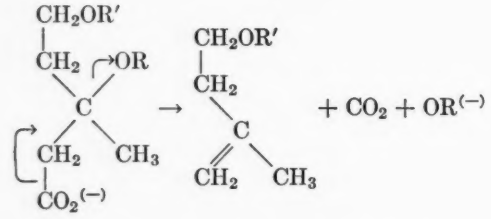
Mevalonate to Squalene. Most of the progress in this section has been made by use of preparations from yeast, the systems in animal tissues offering more resistance to purification. The first three stages happen to be phosphorylations, and have been studied in the laboratories of Bloch and of Lynen; Popják has reported complementary studies with liver preparations.

All three phosphorylations require adenosine triphosphate and a divalent cation, and thus appear to be of a normal type. The enzyme effecting the first step leading to 5-phosphomevalonic acid is called mevalonic kinase and has been partially purified from yeast autolysate (71, 73) and from pig liver (74). It phosphorylates only "natural" (+)-mevalonic acid.

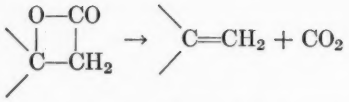
The second phosphorylation apparently produces the pyrophosphate, 5-diphosphomevalonic acid (75); this substance has not yet been well characterized, nor has the product of the third phosphorylation (76). The next identifiable intermediate is isopentenyl pyrophos-

phate (75, 77), $CH_2{=}C(CH_3)CH_2CH_2OP_2O_6H_3$. This has been identified by enzymic hydrolysis to isopentenol, $CH_2{=}C(CH_3)CH_2CH_2OH$, and recently by synthesis (77).

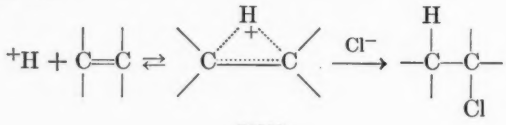
In isopentenyl pyrophosphate, the carboxyl carbon (C-1) of mevalonate (numbering as in XII) has been lost: it had already been shown (78) that this carbon of mevalonate is not incorporated into cholesterol. There is evidence, however, against the process being a simple decarboxylation. Rilling *et al.* (79) studied the biosynthesis of squalene from mevalonate and yeast enzymes in a heavy water medium: 3.5-3.9 atoms of deuterium then entered each molecule of squalene. Now if a simple decarboxylation of a mevalonic acid derivative occurred in this medium, the group $—CH_2—CO_2H$ would become $—CH_2D$. Squalene is certainly built up from some six units of mevalonate, and in the four central units this group $—CH_2D$ would appear as a $—CH_2—$ group linking the next mevalonate unit (80, 81). Thus in these four units one hydrogen atom has been lost from each $—CH_2D$, and in the absence of an isotope effect the average composition of the resulting methylene group would be $—CH_{1\frac{1}{3}}D_{\frac{2}{3}}—$ and the total number of D atoms in squalene $2 + 4 \times \frac{2}{3} = 4.67$, if no further deuterium were introduced during biosynthesis. The discrepancy between this and the experimental value is not conclusive—if small amounts of exchangeable protium were available, they could, aided by the large kinetic isotope effect, depress incorporation of deuterium; and degradation of the deuterated squalene gave succinic acid containing as much as 0.5D, an observation difficult to reconcile with the presence of deuterium at only 3 or 4 positions of the molecule—but if it is accepted as valid, the decarboxylation is not a replacement of $—CO_2H$ by —H but accompanies an elimination of some other group. One such elimination, postulated by Bloch (79), is



Here $OR^{(-)}$ may be a phosphate ion; the process is analogous to the known loss of carbon dioxide and halide ion from some β-halogeno acids in weakly alkaline solution (32). β-Lactones also can lose carbon dioxide to give olefins (66).

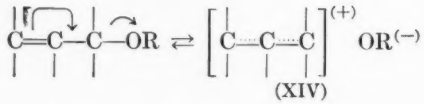


With the formation of isopentenyl pyrophosphate, the biosynthesis enters a series of transformations dominated by the nucleophilic activity of the C=C double bond. Four electrons participate in such a bond, and two of them are readily available for reaction with an electrophilic species, such as a proton or a Lewis acid. The initial attack of, e.g., a proton is thought to produce a charged complex (XIII) which may stabilize itself either by expulsion of a proton (not necessarily the same proton) or by accepting an anion, e.g., chloride ion.

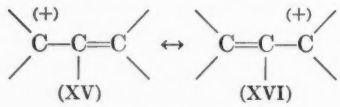


(XIII)

When such a group as halide or hydroxyl or esterified hydroxyl (e.g., a phosphate ester) is on a carbon atom α to the double bond, an elimination can take place rather easily, for the resulting cation (written as XIV) is stabilized by resonance between two equivalent forms (XV) and (XVI).

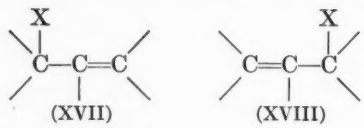


(XIV)



(XV) (XVI)

In this cation, attack of a nucleophilic species $(X^{(-)})$ can be at either end of the three-carbon system, to give (XVII) or (XVIII). Similarly, expulsion of a proton

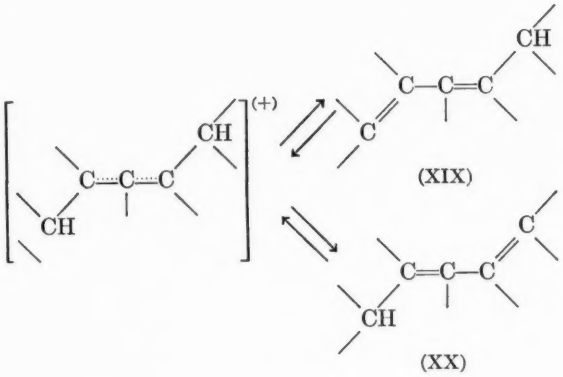


(XVII) (XVIII)

from the cation can also be from either end of the chain, leading to different conjugated dienes (XIX) and (XX).

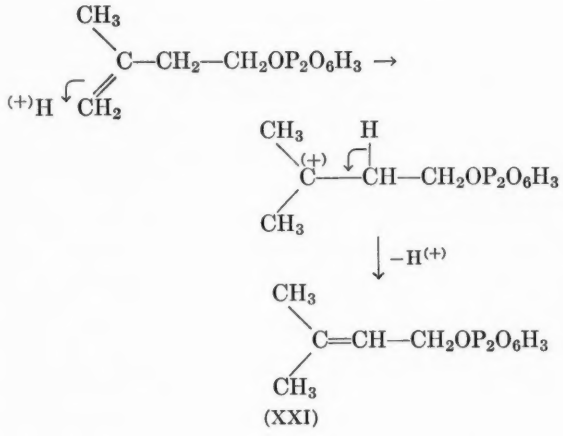
The direct enzymic conversion of isopentenyl pyrophosphate, both natural and synthetic, into squalene has been demonstrated (75, 77). If one accepts the view that six molecules of this substance, as of mevalonate, are required to furnish one molecule of squal-

ene, it follows that a shift in the position of the double bond in each unit must accompany the synthesis. Squalene terminates at both ends in the group $(CH_3)_2C{=}CH$-; thus it is reasonable to suppose that the initial condensation of two $C_{(5)}$ units is preceded by an isomerization of one of them to give 3,3-dimethylallyl pyrophosphate (XXI). This can be done by an addition, of the type discussed above, of a proton to the terminal methylene group, followed by elimination of a different proton.



(XIX)

(XX)

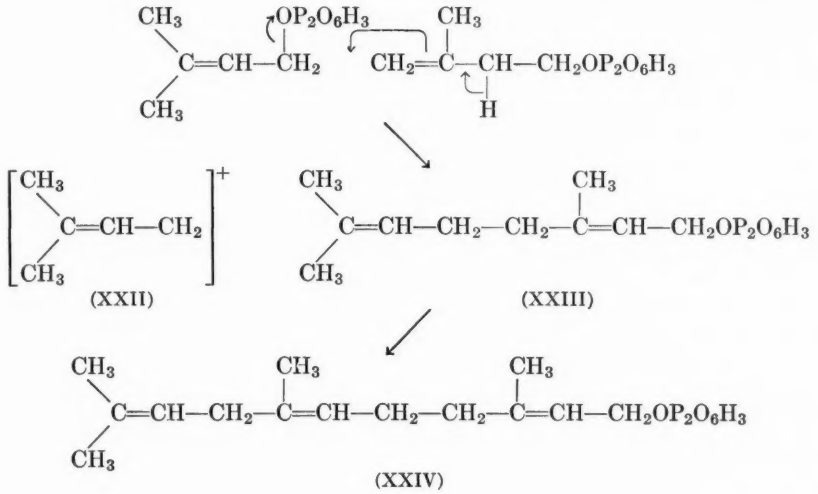


(XXI)

Lynen and his colleagues have recently reported (83) that this transformation can indeed be effected enzymically by a fraction from yeast.

Dimethylallyl pyrophosphate is a substance, of the type discussed above, which can readily give an allylic cation (XXII) by loss of pyrophosphate ion. This cation, an electrophilic agent, can then add to the double bond of isopentenyl pyrophosphate in the same manner as a proton added in the first step; a similar sequence then leads to geranyl pyrophosphate (XXIII), which can itself give a cation and react in the same way with another molecule of isopentenyl pyrophosphate to give farnesyl pyrophosphate (XXIV). The formulae show the electron-shifts without intermediate stages.

The identification of farnesyl pyrophosphate as an intermediate in the biosynthesis of squalene from isopentenyl pyrophosphate is described by Lynen and colleagues (77), who put forward the synthetic scheme formulated above. An essentially similar scheme had previously been proposed by Bloch (84). The condensation may in other biosyntheses proceed to the addition of further $C_{(5)}$ units; this is presumably so with carotenoids (85, 86) and with coenzyme Q (87), both of which can be built up from mevalonate. The biosynthesis of the rubber hydrocarbon (XXV) from synthetic isopentenyl pyrophosphate is said to occur (88) in the latex of *Taraxacum kok-saghyz;* here the

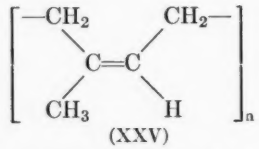


(XXII)

(XXIII)

(XXIV)

double bonds have the opposite geometry from that in geraniol, farnesol, and squalene, a difference which could be the result of different orientation of $C_{(5)}$ units for polymerization.



(XXV)

Farnesyl pyrophosphate (XXIV) is converted (77) into squalene by a particulate fraction of yeast in the presence of reduced triphosphopyridine nucleotide. This process is a reductive coupling of two $C_{(15)}$ units at the carbon atoms bearing the ester groups, and thus has a superficial resemblance to the chemical synthesis of squalene (89) by reaction of farnesyl bromide with lithium. Two mechanisms have been tentatively suggested (76, 77) for the coupling of the $C_{(15)}$ units. In the first, loss of phosphate from both units is assumed, the hydrocarbon farnesene then coupling with uptake either of two protons and two electrons or of one proton and a "negative" hydrogen such as is supplied by reduced pyridine nucleotides. The evidence concerning the participation of "negative" hydrogen is thus far indecisive, DPND introducing only 0.3 atom of deuterium, at undetermined positions, into squalene during biosynthesis from mevalonate. The second mechanism imagines the participation of the reduced form of a quinone, e.g., coenzyme Q. This forms a tertiary ether with one molecule of farnesyl pyrophosphate; a concerted reaction then occurs, liberating the quinone and providing electrons for nucleophilic attack on a second molecule of farnesyl pyrophosphate. The role of the reduced pyridine nucleotide is then to regenerate the hydroquinone, and none of its "negative" hydrogen enters squalene.

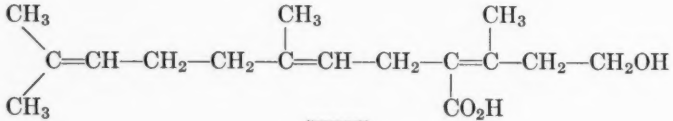
These two mechanisms are illustrated in Scheme 7. Neither of them can at present be supported by chemical analogies; fortunately, the current rapid progress should soon permit more detailed study of this highly interesting coupling. It is worth remarking that a number of chemical reductive couplings, e.g., of ketones and esters, are thought to proceed via free radicals.

Most of the information concerning the stage mevalonate → squalene has so far been obtained by the use of yeast enzymes. The existence of a parallel sequence in mammalian tissues is not yet completely proved. Conversion of mevalonate to squalene in such systems is of course established (80), and the conversion of 5-phosphomevalonate (90) and of synthetic isopentenyl pyrophosphate (91) into cholesterol by preparations of rat liver is also indicated. However, the formation of considerable amounts of higher carboxylic acids from mevalonate in incomplete systems (90) has not yet been explained. An acid recently obtained (92) from such a system was examined chemically (93) and the structure (XXVI) was assigned; it is, however, a poor precursor of unsaponifiable lipids in a complete system.

Squalene to Lanosterol. This cyclization is thought to take place as a single concerted reaction. If this is so, it is the most complex chemical reaction yet known, no fewer than sixteen centers participating. Since the conversion of squalene to lanosterol was originally proved (94), the transformation has been demonstrated (95) with pure, synthetic all-*trans* squalene, and it has been shown (96) that the aqueous medium provides neither oxygen nor stably bound hydrogen to the product. Chemically, the process is oxidative, and O^{18} from the oxygen gas necessarily present during incubation has been shown (96) to enter the hydroxyl group of lanosterol. The cyclizing enzyme has been tentatively named squalene oxidocyclase I.

Despite its complexity, the process depends on a relatively simple property: the nucleophilic activity of a double bond. As explained earlier, the electrons available at a double bond can attack an electron-deficient species, say $X^{(+)}$. The product (XXVII) is itself electron-deficient and can attack another double bond to give a new species (XXVIII); and if this second double bond is in the same molecule, the process is a cyclization.

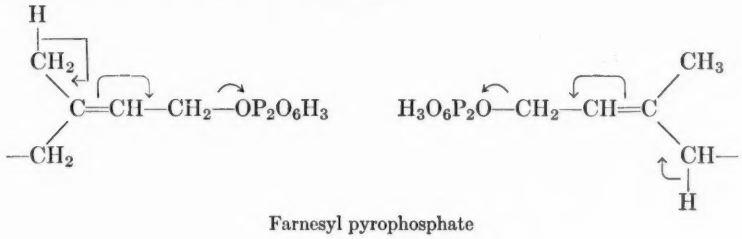
"Carbonium ions" such as (XXVII) or (XXVIII) are highly unstable intermediates, and their presence can rarely be demonstrated unless exceptional stabilizing structures are also present. Indeed, the sign (+) on (XXVII) and (XXVIII) may mean no more than a developing electron-deficiency which induces the next step in a concerted reaction. This next step can be any of three types, all of which are found in the cyclization of squalene: (i) reaction with a new nu-



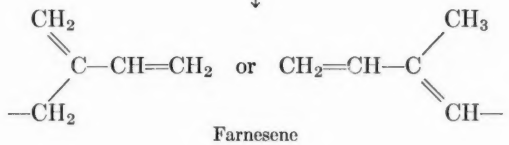
(XXVI)

SCHEME 7

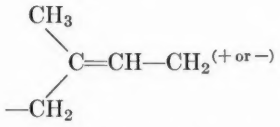
(*a*)

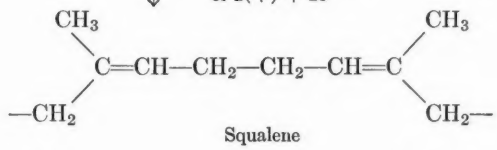


Farnesyl pyrophosphate



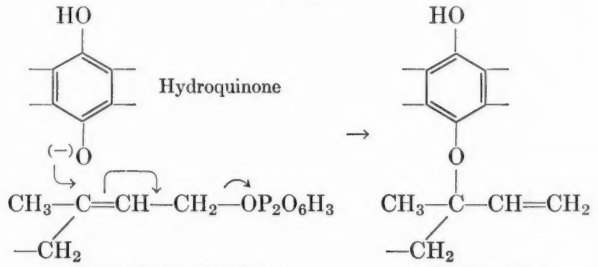
Farnesene





Squalene

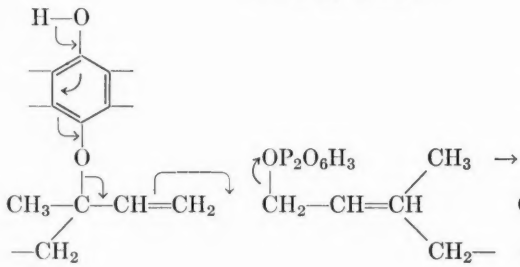
(*b*) (i)



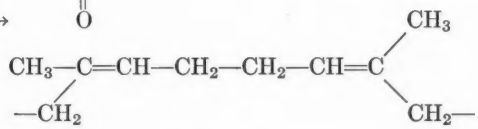
Farnesyl pyrophosphate

Tertiary ether

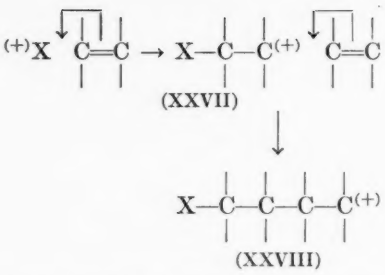
(ii)



Quinone

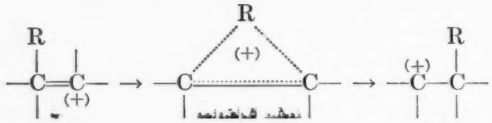


Squalene



(XXVII) (XXVIII)

cleophilic species; (ii) elimination of a cation, usually a proton, to give a neutral molecule; (iii) a *1,2-shift* transferring the electron-deficiency to an adjacent carbon, from which a group migrates (XXIX → XXX).



(XXIX) (XXX)

A detailed theory of the chemical change leading from squalene to cholesterol has been formulated by the Zürich school (97). It is possible to fold the long squalene molecule in such a way that an electron-shift, assumed to be initiated by an electron-deficient oxygen atom such as ^{+}OH, can be transmitted in a concerted manner through five double bonds (Scheme 8). A tetracyclic system is thus formed, with an electron-deficient center at the carbon atom which is to become C-20 of lanosterol. A series of 1,2-shifts then occurs; four groups migrate, and the electron-deficiency thus transferred back to the junction of the two central rings is finally eliminated by expulsion of a proton from C-9.

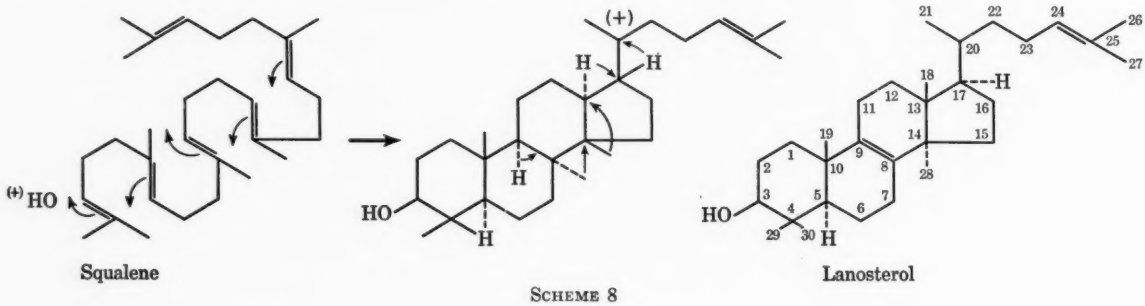
The Swiss authors showed that by slightly different foldings of the squalene molecule, and slightly different sequences of the same types of nucleophilic attack and rearrangement, it was possible to formulate all known types of triterpenes as being derived from squalene. This is the most elaborate formulation of Ruzicka's "isoprene rule," which has helped in the interpretation of so many terpenoid structures and has been vindicated by the discovery of the basic building unit isopentenyl pyrophosphate, a substance formally derivable from isoprene by addition of pyrophosphoric acid. The mode of cyclization is supported by the labeling patterns found by total degradations of squalene (98) and of cholesterol (99 to 103) biosynthesized from acetate. The proof (95, 105) that both the angular methyl groups 18 and 28 in lanosterol have indeed reached their positions by 1,2-shifts has provided further detailed support for the mechanism proposed.

A concerted cyclization of the type illustrated can be regarded, as explained above, as a series of nucleophilic attacks by electrons of double bonds on electron-deficient centers, and it is not logically necessary to assume that these should all occur simultaneously. The novelty of the process, chemically speaking, is the stereochemical control, eight new centers of optical asymmetry being fixed in their proper mutual relationships when cyclization is complete. Attempts to achieve similar cyclizations nonenzymically have had some success, e.g., the transformation (XXXI → XXXII) effected by Eschenmoser (105) in 5 to 10 per cent yield.

The available evidence is against a concerted mechanism for such multiple cyclizations in solution, but given a surface, such as the enzyme presumably provides, for attachment of the substrate in the most favorable configuration, a concerted reaction might well occur. By contrast, when a rigid system of carbon rings is provided and the necessary centers are already fixed in proper relationship, concerted *rearrangements* quite as complex as that leading to lanosterol can be achieved nonenzymically, e.g., (106), (XXIII → XXXIV).

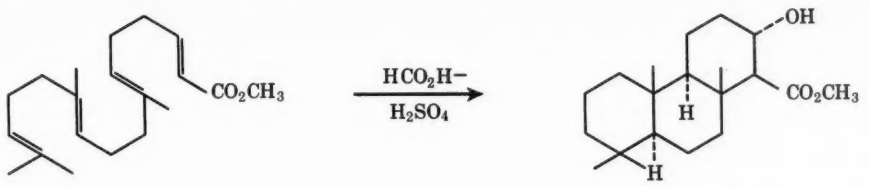
Lanosterol to Cholesterol. Since the original demonstration of the over-all process (107), the course of the transformation has been deduced in more detail (see Scheme 5).

The necessary removal of three methyl groups is an

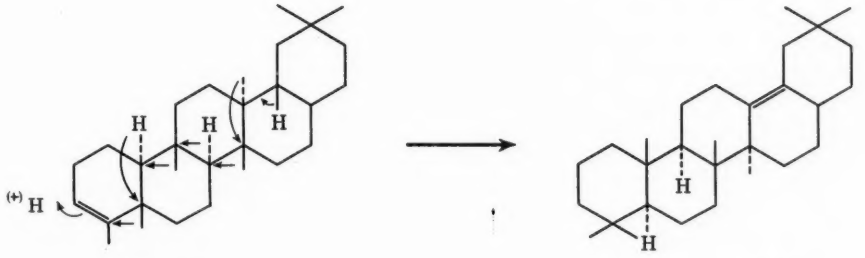


Squalene Lanosterol

SCHEME 8



(XXXI) (XXXII)



(XXXIII) (XXXIV)

oxidative process. Studying the conversion, by a particulate preparation of rat liver, of lanosterol to cholesterol, Olson *et al.* (108) found that aerobic conditions were necessary, that the three carbon atoms eliminated appeared as carbon dioxide, and that formaldehyde could not be demonstrated as an intermediate. This lends support to the view that the actual eliminations are decarboxylations.

Oxidations of the sterol skeleton at specific positions necessarily takes place in the biogenesis of, e.g., bile acids, adrenal hormones, and many other types, and similar specific oxidations of steroid substrates artificially supplied can be effected by micro-organisms. It is convenient to consider these oxidations in conjunction with the oxidative demethylation of lanosterol.

Enzymic oxidations of this sort are difficult to classify chemically, because of the lack of analogous non-enzymic processes. When one uses in homogeneous solution an oxidizing agent powerful enough to attack nonactivated C—H bonds at an appreciable rate, it is rare for the product to escape further extensive oxidation. How an enzyme can attack these bonds while leaving untouched more sensitive groups in the same molecule is still unknown, but the mechanism must surely depend on the structure of the enzyme-substrate complex: the substrate may be attached to the enzyme in such a way as to leave exposed to an oxidizing agent only the group to be oxidized, or the enzyme itself may carry an oxidizing group correctly oriented to oxidize one part of the attached substrate and no other.

Three types of chemical mechanism can be envisaged for the direct replacement of a hydrogen by hydroxyl: nucleophilic, homolytic, and electrophilic. In a nucleophilic mechanism, the hydrogen would be removed along with both its bonding electrons and would be replaced by an hydroxyl ion. Since it has been shown (109) that isotopic oxygen from the atmosphere, not from water, provides the 11β-hydroxyl group introduced by adrenal 11β-hydroxylase, and hydroxyl groups introduced at several other positions by micro-organisms (110), this type of mechanism can probably be excluded. In addition, this and other experimental evidence (111) also excluded an indirect hydroxylation via an olefin first formed by dehydrogenation.

By labeling specific hydrogen atoms in various steroids with tritium or deuterium it has been possible to show that the 11β-hydroxylation of progesterone by perfusion through bovine adrenals (112), the microbiological 11α-hydroxylation of pregnane-3,20-dione (112, 113), and the 7α-hydroxylation occurring during biosynthesis of cholic acid from cholesterol in the living rat (114), all proceed by direct replacement of hydrogen by hydroxyl without change in configuration. It has been pointed out (113, 114) that this retention of configuration is consistent with what little is known of the stereochemistry of electrophilic displacements at a saturated carbon atom. The evidence therefore favors the view that the attacking species is electrophilic, like the $^{(+)}$OH postulated as the initiator of squalene cyclization, and that the departing hydrogen

leaves behind the electrons which bound it to carbon. A homolytic mechanism, with free radicals as intermediates, is not excluded by the evidence but requires additional assumptions to explain retention of configuration.

Oxidation of the three methyl groups eliminated during conversion of lanosterol to cholesterol evidently proceeds farther than hydroxylation, and the evidence is clear that the methyl group C-28 is eliminated first. The ingenious demonstration (115, 116, 117), that 4,4-dimethylcholesta-8,24-dien-3β-ol is an intermediate, overcame great difficulties occasioned by the minute amounts of available radioactive material. An interesting feature of the elimination of the two methyl groups at C-4 is the demonstration that lanosterol labeled in the 3α position with tritium loses the isotope completely on the way to cholesterol, whereas zymosterol similarly labeled does not; and that 4,4-dimethylcholesta-8,24-dien-3-one is converted enzymically into cholesterol as efficiently as is the corresponding alcohol, whereas lanostadienone, the ketone of lanosterol, is inert. As Bloch (84) pointed out, oxidation to carboxyl of the 4-methyl groups in a 3-oxo-4,4-dimethyl-steroid would give β-keto acids which would decarboxylate with ease. In comparison, any assistance given by the 8,9-double bond in lanosterol to decarboxylation of a 14-carboxylic acid would be rather slight.

The first $C_{(27)}$ intermediate appears to be zymosterol (118, 119) or possibly the corresponding ketone. Of the transformation of this into desmosterol (120)—for formulae see Scheme 5—or of the mechanism of reduction of the 24,25-double bond, nothing is at present known. Chemically, neither process is without analogy, and light can perhaps be thrown on the mechanism by suitable labeling of hydrogen atoms.

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Scintillation counter for the measurement of radioactivity of vapors in conjunction with gas-liquid chromatography

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SUMMARY

The construction and performance of a liquid scintillation counter for the measurement of radioactive vapors is described. The instrument is used in conjunction with a gas-liquid chromatographic instrument for the measurement of C^{14} contained in chromatographic vapors. With a single photomultiplier tube 50 per cent of the C^{14}-disintegrations are counted and recorded parallel with the chromatographic analysis. The records obtained are of the integral type. The resolving power of the counter is such that radioactive fractions needing only 25 seconds to pass through the chromatographic analyzer (gas-density balance) may be clearly distinguished from other radioactive fractions following in 1 minute or less.

The instrument described in this paper was designed to measure the radioactivity of C^{14}-fatty acids emerging as vapors from a gas-liquid chromatographic column and to record the radioactive disintegrations parallel with the analytical chromatographic record. The need for such an instrument arose out of work in this laboratory on the biosynthesis of fatty acids and other volatile substances from C^{14}-labeled precursors in the course of which a resolution of mixtures of the substances and assay of the C^{14}-content of the individual components became necessary. To accomplish such a task by available methods proved time-consuming and in some instances misleading or inaccurate. Paper chromatography of fatty acids has limited powers of resolution; liquid-liquid partition chromatography (e.g., on a reversed phase column) offers a wider scope, but a complete analysis of a complex mixture together with assay of isotope in each chromatographic fraction may take a week or longer. The gas-liquid chromatographic technique of James and Martin (1) is undoubtedly the method of the greatest power of resolution for volatile substances. But even in this method, if a mixture of C^{14}-labeled fatty acids (or their methyl esters) are resolved, the collection of the fractions followed by plating of the samples for radioactive assay presents problems, not the least among which is the loss by volatilization of the methyl esters of acids with a short or medium-length carbon chain from planchettes. Collection of fractions by bubbling the effluent vapors and carrier gas from a gas-liquid chromatographic column directly into a solution of scintillator, followed by counting in a suitable scintillation counter, has been used by us with success; the method is simple and sensitive but lacks resolution. It may be used only when the retention volume of each radioactive chromatographic fraction is known precisely.

Specimens may be encountered in which a radioactive component of high specific activity may be present in such small quantities that even the gas-density balance, one of the detectors used in gas-liquid chromatography (2), may not show them distinctly or such components may overlap with some well-recognized fatty acid. In such instance the radioactivity may erroneously be attributed to the wrong substance. For these reasons it was thought desirable to develop an instrument that will record continuously the radioactivity of vapors in conjunction with gas-liquid chromatography. It will be seen from the records presented that the power of our instrument to differentiate between radioactive fractions emerging from the gas-liquid chromatographic instrument is as great as the

power of the columns employed; the radioactivity of a fatty acid ester that takes only about 20 to 30 seconds to pass through the analytical detector (gas-density balance) may be correctly assigned to that ester and is clearly separated from another ester emerging 1 minute or less afterwards. The principle of the instrument and a simple prototype has already been described briefly (3).

PRINCIPLE OF THE INSTRUMENT

The principle of the instrument is very simple and depends on the injection of the chromatographic vapors together with the carrier gas (N_2) into a continuously circulating and cooled solution of phosphor (diphenyloxazole in toluene or xylene). The organic vapors, by virtue of their solubility in the solvent and their high boiling point, become condensed and dissolved in the toluene or xylene; the carrier gas, on the other hand, escapes to the atmosphere. If the substance condensed in the solution of phosphor is radioactive, then the light emission (scintillation) excited by the radiation can be measured with a photomultiplier tube.

Let us imagine a circuit consisting of tubes *A*, *B*, *C*, and *D* as shown in Figure 1; at the junction of *D* and *A* there is a capillary inlet (*I*) and at the junction of *A* and *B* a chimney (*E*) open to the atmosphere. If this circuit were filled with liquid, this would leak out through the capillary *I*, but if gas is blown through the capillary, the gas pressure prevents this: the gas bubbles rise in tube *A* and escape through *E*. Since the gas bubbles displace liquid in tube *A*, the liquid will circulate in the direction of the arrows (Fig. 1). When the circuit is made of tubing with an internal diameter of 4 mm. and gas is blown through *I* at the rate of 40 to 160 ml. per minute (gas flow rates commonly used in gas-liquid chromatography) the circulation of liquid is very vigorous, as can be shown by the addition of dyes to the liquid; complete mixing takes place in our final instrument within 5 seconds when a gas flow of 40 ml. per minute is used. If the liquid in the circuit is a solution of a phosphor (liquid scintillator) and radioactive vapors are injected at *I*, the radioactive material will pass around the circuit and excite the emission of light photons, which can be measured with a photomultiplier placed at the junction of tubes *B* and *C* (indicated by the dotted circle in Fig. 1). This is the prototype of our instrument, which was constructed and found to function correctly (3). The output from the photomultiplier, after amplification, is measured with a ratemeter and continuously recorded. Since the radioactive material accumulates in the solution of phosphor, the record obtained is of the integral type, a distinct advantage over the usual type of chromatographic record.

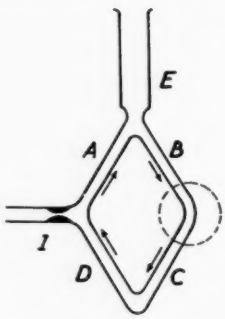
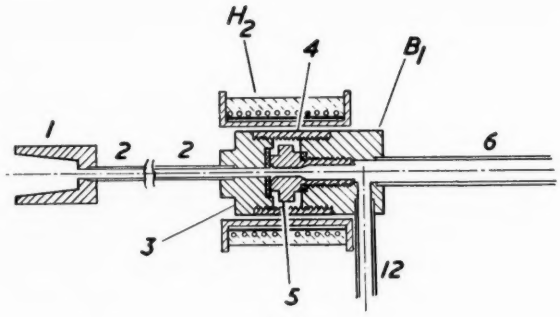


FIG. 1. Schematic representation of principle of scintillation counter for measuring radioactivity of vapors. For description see text.

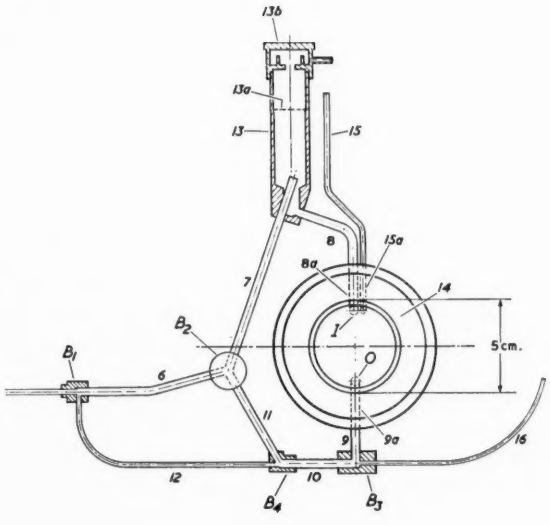
DESCRIPTION OF THE INSTRUMENT

In the construction of the instrument several conditions had to be met: (*a*) the solution of the scintillator and photomultiplier had to be kept cold, while the chromatographic vapors—in order to avoid their condensation before they reach the scintillator—had to remain at the temperature (up to 200°C) of the chromatographic instrument up to the point of their injection into the scintillator; (*b*) in order to obtain maximum efficiency there must be no light-absorbing surfaces interposed between the scintillator and the window of the photomultiplier and the whole window area of the photomultiplier must be used "to see" the scintillations; the photomultiplier must see the largest possible fraction of the circulating scintillator; (*c*) gas bubbles must not appear in front of the photomultiplier, as these would prevent some of the light photons—by internal reflections—from reaching the sensitive photocathode; and (*d*) the instrument must be light-tight. The prerequisite to all these conditions, an efficient removal of organic vapors from the carrier chromatographic gas and rapid mixing in the scintillator, was assured from our preliminary experiments with a prototype instrument.

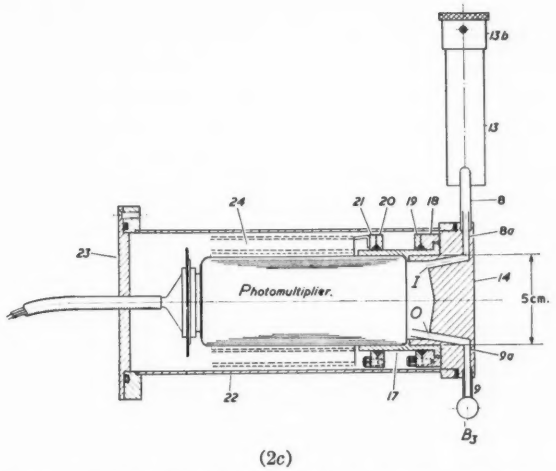
Condition (*a*). In order to prevent condensation of the organic vapors before they are injected into the scintillator, the vapors and carrier gas are conducted from the detector (gas-density balance) of the gas-liquid chromatographic instrument to the counter through an electrically heated tube, which is kept 10°-20°C above the temperature of the gas-density balance (2). The whole instrument, which has a large heat capacity, is kept inside a refrigerated box (−5°C);



(2a)



(2b)



(2c)

FIG. 2. Constructional details of scintillation counter for measuring radioactivity of vapors. Components shown on more than one illustration are given the same numbering: (*a*) heated tube connecting counter with gas-liquid chromatographic instrument and injection nozzle; (*b*) cross-section of counter through counting chamber; (*c*) side elevation of counter. For description of individual components see text. Figures 2*b* and 2*c* were drawn to scale.

only the heated connecting tube and the point of injection of the vapors is outside the cold box.

Condition (*b*). In order that the photomultiplier may see the largest possible fraction of the circulating scintillator, the portion of the circuit between tubes *B* and *C* shown in Figure 1 was expanded into a shallow circular chamber of very nearly the same diameter as the diameter of the photomultiplier tube. The volume of this chamber is about 18 ml.; the total volume of scintillator required to fill the instrument is 25 ml. and therefore the photomultiplier sees 72 per cent of the circulating liquid. To avoid light-absorbing surfaces between scintillator and photomultiplier, the window of the photomultiplier acts as the front wall of the circular chamber.

Condition (*c*). To avoid loss of light photons reaching the photomultiplier by internal reflection of light within bubbles, the gas and vapors are injected into the scintillator outside the "counting chamber"; but even so, when a high rate of gas flow is used in the chromatography, the circulation of liquid in the counting instrument is so vigorous that the stream of fluid may drag gas bubbles from the escape funnel (Fig. 1, *E*) into the counting chamber and eventually a gas-lock may develop at the top of the counting chamber. This has been prevented by placing tube *A* (which corresponds to tube 7 in Fig. 2*b*) at a slope, rather than vertically, and by extending it into the chimney (*E*) to a height about 2.0 cm. above the level of the orifice of tube *B*. Further, a vent pipe has been inserted into the top of the counting chamber, through which gas dragged into this chamber may escape, or may be sucked out with a syringe (see "Operation of the Instrument" below).

Condition (*d*). With the exception of the photomultiplier and the polyethylene sleeve used for its attachment (see below), the functional parts of the instrument have been made of stainless steel and the photomultiplier tube is sealed in a light-tight and moisture-proof metal casing.

CONSTRUCTION OF THE INSTRUMENT

The construction of the instrument is illustrated in Figures 2*a*, 2*b*, and 2*c*, and its assembly in Figure 3. The counter is connected to the detector of the gas-liquid chromatographic instrument through a stainless steel tube (1.5 mm. bore; 15 cm. long; Fig. 2*a*; com-

ponent 2), which at one end is hard soldered into a brass socket of a standard taper joint (Fig. 2*a*; component 1), and at the other end into the hole of a screw provided with a hexagonal head (Fig. 2*a*; component 3). The socket fits the cone, which is the outlet of the chromatographic detector, and the screw fits into the sleeve (Fig. 2*a*; component 4), which surrounds the injection nozzle. Before the connecting tube is soldered into the brass socket, a woven-glass insulating sleeve is pulled over the tube. As this insulating sleeve can be stretched or compressed, the soldering can be done without damage to it. An insulated copper-constantan thermocouple is pushed under the insulating sleeve over which a heating element H_1 (not shown on Fig. 2*a*) is wound out of nichrome wire (30 standard wire gauge) insulated with woven-glass sleeving. The total resistance of this heater is 4 ohms.

The connecting tube leads to the injection nozzle fitted to block B_1 (cf. Figs. 2*a* and 2*b*; component 4), the construction of which is shown in detail on Figure 2*a*. The actual nozzle is a 6BA screw with a hexagonal head and drilled out as illustrated (Fig. 2*a*; component 5); it is screwed into block B_1. Copper gaskets are used in front and at the back of the injection nozzle to ensure gas-tightness.

The chromatographic vapors enter the scintillator, circulating in tubes 6 to 12 and the counting chamber, through the 1 mm. bore hole of the nozzle. This particular construction of the injection nozzle (and of block B_1) was arrived at after testing four other simpler designs, all of which proved unsatisfactory mainly because the scintillator tended to crystallize out around the capillary inlet, causing blockage which was difficult to clear or because the capillary inlet could not be heated adequately to prevent early condensation of vapors. The present arrangement permits quick dismantling and cleaning of the injection nozzle and connecting tube, although this has not been necessary during more than 6 months of daily operation. Also we had no sign of tailing of radioactive peaks that could be attributed to premature condensation of vapors, whereas such troubles were met in earlier designs.

Before the connecting tube is screwed into the sleeve (Fig. 2*a*; component 4) surrounding the injection nozzle, an electric heater (H_2) wound on a metal reel is fitted over the sleeve and heats block B_1 and the parts of the connecting tube that screw into B_1. This heater, like that for the connecting tube, is made, over woven-glass insulation, from nichrome wire (30 standard wire gauge; 6 ohms). A thermocouple is inserted under the insulation of this heater also. The heaters are supplied with current from suitable tappings on two low-voltage transformers. These transformers are fed from a duplex variable transformer.

The liquid scintillator circulates in tubes 6 to 12 (Fig. 2*b*); the circuit includes the counting chamber 14. These tubes are joined together by hard-soldering into four stainless steel blocks B_1 to B_4, into the gas escape chimney 13 and into the block of the counting chamber 14, all drilled at the appropriate angles. The liquid scintillator leaving tube 10 divides into two streams, into tubes 11 and 12. Tube 12, a bypass of the main stream of scintillator, carries liquid just to the tip of the injection nozzle and has an internal diameter of 2 mm., whereas all the other tubes (6 to 11) are of 4 mm. internal diameter. This bypass has been introduced in order that the injection of hot vapors may be made away from the cooled parts of the instrument and in order that all the circulating phosphor may not be heated; in Figure 2*b* the parts to the right of block B_1 are inside and those to the left are outside the refrigerated box.

The gas-escape chimney (Fig. 2*b*; component 13), 7.5 cm. long, internal diameter 1.6 cm., is machined out of a solid rod and has a narrower well (8 mm. diameter, 1.5 cm. deep) at its bottom. The holes, into which tubes 7 and 8 are soldered, open into this well; tube 7 protrudes into, and slightly beyond, the well to a total length of 2 cm. Thus the orifice of tube 8, in which liquid moves downward, is below the level of the scintillator fluid where the gas bubbles escape from tube 7. The arrangement prevents gas bubbles from being dragged into the counting chamber. Inside the chimney, 2 cm. from the top, a fine platinum wire mesh soldered to a ring (component 13*a*) has been inserted in order to break up the splashing of the scintillator. The chimney is covered with a screw-cap lid (component 13*b*) on the inside of which there is a simple light trap. A side tube screwed into the cap allows the carrier chromatographic gas to escape and is usually connected through a polyethylene tube to a gas-flow meter.

The block of the counting chamber 14, to which the photomultiplier tube is attached, is machined on a lathe from a stainless steel disk 8.5 cm. in diameter and 3.6 cm. thick. The internal diameter of the counting chamber is 4.4 cm., its outer diameter 5 cm., and its depth at the side is 8 mm. The bottom of the chamber has been machined to the shape of a shallow cone and highly polished to provide a reflecting surface for light. The inlet and outlet for the scintillator fluid is provided by the channels (4 mm. diameter) (Figs. 2*b* and 2*c*; components 8*a* and 9*a*) drilled to form an elbow at the upper and lower edges of the chamber. A polyethylene tube has been inserted tightly into the

exit hole *O*, and protrudes into the counting chamber by 8 mm. The insertion of this tube ensures that the liquid entering through the inlet hole (*I*) will not stream down the bottom of the counting chamber, but will mix with its contents. At the upper edge of the counting chamber and parallel with the inlet channel (component 8*a*) there is a similar size channel (component 15*a*) which opens into the vent pipe (Fig. 2*b*; component 15). A stiff polyethylene tube with a slight upward curvature is inserted into the orifice of this ventilating channel and reaches to the front upper corner of the counting chamber and allows the air to escape from the counting chamber during filling of the instrument with scintillator (see also "Operation of the Instrument" below). The counter is filled and emptied through tube 16 (Fig. 2*b*), internal diameter 2mm., which is soldered into block B_3.

The polyethylene sleeve (Fig. 2*c*; component 17), 3 mm. thick, which secures the photomultiplier to the counting chamber has a 2 mm. thick internal ridge which sits on the edge of the wall of the counting chamber. This ridge acts as a cushion between the glass photomultiplier and the metal edge of the counting chamber and prevents damage to the tube during assembly. Thus the depth of the counting chamber at its side walls is 10 mm. The polyethylene sleeve is secured by its flange to the chamber block with a ring (Fig. 2*c*; component 18) screwed to the block. Although polyethylene does not dissolve in either toluene or xylene (solvents for the scintillator), it swells slightly on prolonged exposure to these solvents. In order to assure a tight fit of the sleeve around the counting-chamber wall and the photomultiplier, O-rings have been fitted around both and squeezed tightly between the V-grooves formed by two rings appropriately shaped (Fig. 2*c*; components 18, 19, 20, and 21). No leakage of scintillator has been observed with this sealing arrangement over several months of operation. It is seen that the window of the photomultiplier tube forms the front wall of the counting chamber; thus the only loss of light is by absorption in the scintillator itself.

A light-tight cover (Fig. 2*c*; component 22), made of a brass cylinder and painted black, fits over the photomultiplier. It is secured to the block of the counting chamber by a screw thread and is sealed off with a rubber gasket. This cover is fitted with a lid (Fig. 2*c*; component 23) which has a hole in its center for the cable to the photomultiplier. A rubber sleeve over the cable ensures a light- and air-tight fit.

A double-walled cylinder made of perforated tin sheet and lined with filter paper (Fig. 2*c*; component 24) is filled with silica-gel drying agent and placed around the photomultiplier before the light-tight cover is screwed down.

The whole counter assembly is surrounded by a lead shield, 2.5 cm. thick (Fig. 3). The lead shield was

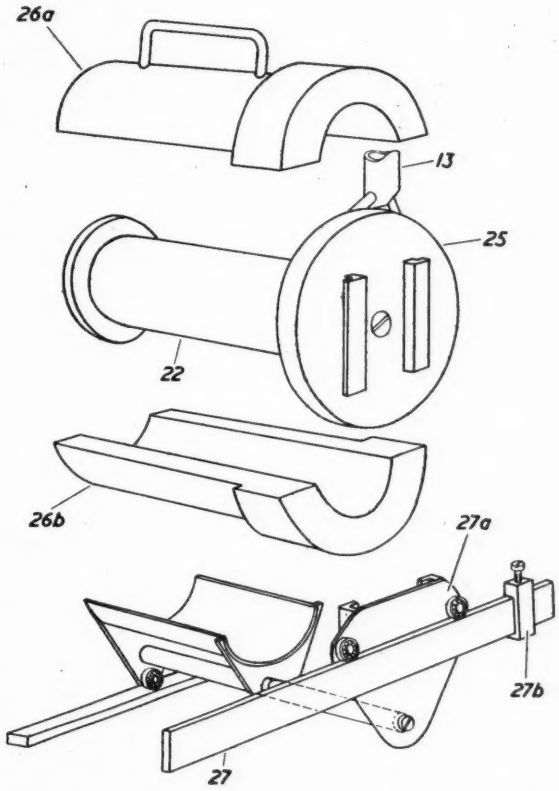


FIG. 3. Components of lead-shield and cradle for scintillation counter.

made in three parts: (*a*) a disk to which the counting chamber is secured with a stout screw; this lead disk (Fig. 3; component 25) is provided with a handle (not shown in Fig. 3) and two vertical slides which secure the counter to a cradle (see below) inside a refrigerated box; and (*b*) a cylinder slit into two halves along its length (Fig. 3; components 26*a* and *b*). As the photomultiplier lies horizontally, the lower half of the lead cylinder acts as a cradle for the instrument and is mounted inside a refrigerated box on sliding rails (Fig. 3; component 27). It is the sliding-rail arrangement of this cradle which carries a vertical plate (component 27*a*) to which the counter assembly is secured by the vertical slides attached to the lead disk described above.

When the counter is fitted into the refrigerated box, the cradle is rolled to the back of the box and the

counter, secured to the lead disk, is lowered into the cradle; the top half of the lead shield is then put into position. The whole assembly is then rolled forward until the injection nozzle protrudes out of the refrigerated box through a hole cut into the front of the box; the counter is now secured in this position by tightening the clamping screw of the sliding arrangement (Fig. 3; component 27*b*) inside the box. The cylindrical electric heater (H_2) is now pushed over the injection nozzle and the tube connecting the counter to the gas-density balance is screwed tightly into the sleeve surrounding the injection nozzle.

The arrangement of the sliding cradle has been made in order that the counter may be placed quickly and easily into a predetermined position inside the refrigerated box and without causing damage to the protruding pipes joined in block B_1. The aim in the construction of the lead shielding and cradle—as of other parts too—has been an ability to dismantle the instrument quickly if a need for maintenance should arise.

The refrigerated box, which houses the counter, measures $43 \times 43 \times 43$ cm. on the outside; the thickness of its insulated walls is 7 cm. The cooling coil on the inside of the box is connected to a single-stage compressor unit through flexible metal tubing. The thermostat to the refrigeration unit is set to $-5°C$ and responds to the air temperature in the box. With this setting, the temperature of the counter and of the scintillator liquid is maintained steadily at 0°C while the hot vapors and carrier gas are injected into it.

The box rests in an angle iron frame on two rollers and can be moved forward and backward on these with a worm gear. The box rests at such height that the socket of the connecting tube is opposite the outlet cone of the gas-density balance; by the use of the worm gear it can be quickly and easily attached to it, or disconnected from it.

The layout of the whole assembly is shown in Figure 4. The output from the photomultiplier tube, after amplification, is measured with a ratemeter.[1] This ratemeter was modified so as to provide for purposes of recording two ranges in the ratio of 10×10^n:3×10^n cps., where n may be selected with a switch to be 0, 1, 2, 3, or 4. Both the $10\times$ and $3\times$ ranges are simultaneously recorded in our instrument and provide two levels of sensitivity for the measurement of radioactivity. The range of the instrument has been further extended by the addition of an automatic switch: when the output from the ratemeter reaches four-tenths of the level of any of the 10×10^n ranges, and therefore the output is beyond the recording limits of the 3×10^n range, the switch automatically changes this to the $3 \times 10^{(n+1)}$ range. A further automatic switch provides protection for the 10×10^n range by disconnecting it from the ratemeter when its reading exceeds 30 per cent overload. As a consequence of this arrangement of recording it is hardly possible to meet a situation when a correct record of the counting rate would not be made. These operational details will be further illustrated by actual records shown in Figures 6 and 7. The records are taken with a three-channel recorder: two of the channels are used for the radioactive record and the third for the analytical chromatographic record, which registers the response of the chromatographic detection device, which in our instrument is a modified gas-density balance which will be described in a future communication.

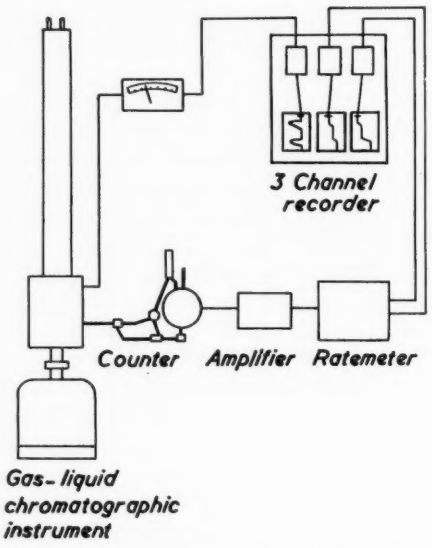


FIG. 4. General layout of scintillation counter, gas-liquid chromatographic apparatus, and recording instruments.

OPERATION OF THE INSTRUMENT

For setting up the counter for measurements the refrigerated box containing the counter is moved forward on its rollers until the socket of the connecting tube fits snugly over the cone of the outlet of the chromatographic instrument through which the chromatographic gas is already flowing at the required rate. A simple manifold, fastened to one of the sides

[1] Type 1172, EKCO Electronics, Ltd., Southend-on-Sea, England.

of the refrigerated box and consisting of three 3-way taps in a line, a syringe, and three reservoirs, provides an easy means for filling the counter with scintillator solution, for removing the used scintillator, and for washing out the instrument. The arrangement is shown in Figure 5. Reservoir R_1, holding the solution of scintillator, is kept inside the refrigerated box; R_2 and R_3 are outside this on a shelf under the box. For filling the counter, taps T_2 and T_3 are turned so as to open the reservoir of scintillator solution (R_1) to syringe S_1; 25 ml. of scintillator solution are now drawn into the syringe and tap T_3 is turned off by rotating it by 45°. Tap T_2 is now turned around by 180° and tap T_1 is turned so as to open the manifold from the syringe to the counter-filling tube. The contents of syringe S_1 are now emptied into the counter. It will be realized that at the moment when the level of the scintillator in the counter reaches above the level of the gas-vapor injection nozzle, the gas flow will force the scintillator liquid up in tubes 6 and 7 into the gas-escape chimney from where the liquid will flow through tube 8 into the counter chamber (cf. Fig. 2*b*). Without the vent pipe 15 it would not be possible to fill the counter chamber completely with scintillator because the flow of liquid from the gas-escape chimney down through tube 8 would prevent the escape of air from the counter chamber. When all the scintillator has been expelled from syringe S_1, any air that might have been trapped at the top of the counting chamber is drawn out with syringe S_2, which is attached to the vent pipe of the counter chamber with a polyethylene tube. The hollow piston of syringe S_2 has two holes, one at the bottom and one at the top; when the air is being drawn out of the counting chamber, the hole at the top of the piston is closed with the tip of a finger. The sign of complete withdrawal of all air from the counter chamber is when a solid column of liquid, uninterrupted by any gas bubbles, appears below syringe S_2. Now by lifting the tip of the finger from the hole of the piston, the liquid drawn up into S_2 will return to the counter. After this manipulation the counter can be operated for several hours without a gas lock developing in the counter chamber. Before the chromatographic run can be started, the electric current for the two heaters, one around the connecting tube and the other around the injection nozzle, are turned on and the variable transformers controlling these currents are so adjusted that the thermocouple reading of the temperature of the connecting tube registers about 20°C above the temperature of the chromatographic instrument and that of the heater for the injection nozzle at about the boiling point of the scintillator solvent (toluene; 110°C). With the heaters we use, the appropriate heater currents are obtained at 6 to 8 V for the connecting tube heater and with 12 to 14 V for the injection-nozzle heater when the chromatography is done at 200°C.

Since during the filling of the counter the scintillator is exposed briefly to light, there is some phosphorescence induced which requires a few minutes to decay. A 5- or 10-minute record is therefore taken before the start of a chromatographic run in order to obtain the stable background level of counting.

With the particular type of photomultiplier employed,[2] it was found that an E.H.T. setting of 1600 V

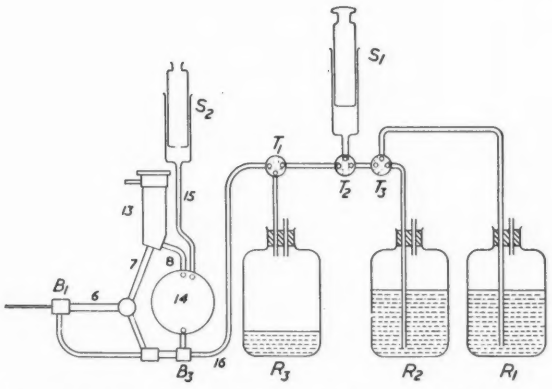


FIG. 5. Manifold used for filling and emptying scintillation counter. For details see text.

to the photomultiplier, together with a discriminator bias voltage of 25 to 30 V on the ratemeter, provided the optimum conditions for counting: the background plus photomultiplier noise being 7 to 10 cps. and the counting efficiency about 50 per cent. It should be pointed out that in the calculation of efficiency no allowance was made for the fact that the photomultiplier can "see" only 72 per cent of the scintillator.

It follows from the background counting level that the lowest useful and most sensitive range that may be selected on the ratemeter for recording purposes is the 100:30 ($10 \times 10:3 \times 10$) setting. In order to obtain a record of radioactivity with the minimum of delay after the entry of a radioactive substance into the counter, the integrating time constant of the ratemeter must be kept short: 1 second in the counting ranges of 100:30 cps. and 0.2 second at all higher ranges should not be exceeded.

[2] VMP 13/44 (Twentieth Century Electronics, Ltd., New Addington, Surrey, England).

On account of these short time constants, the records obtained show the oscillations characteristic of the randomness of radioactive disintegrations. However, these oscillations are around a mean value that can be read without any difficulty (cf. Fig. 6). Only after the background count has settled to a steady level is the sample to be analyzed put on the chromatographic column. During the loading of the column the stream of gas through the counter must be maintained, otherwise the scintillator would flow out through the connecting tube. As our instrument is used in conjunction with a gas-density balance, during the loading of the sample column the gas stream through the reference column is kept flowing and maintains the circulation of the scintillator in the counter.

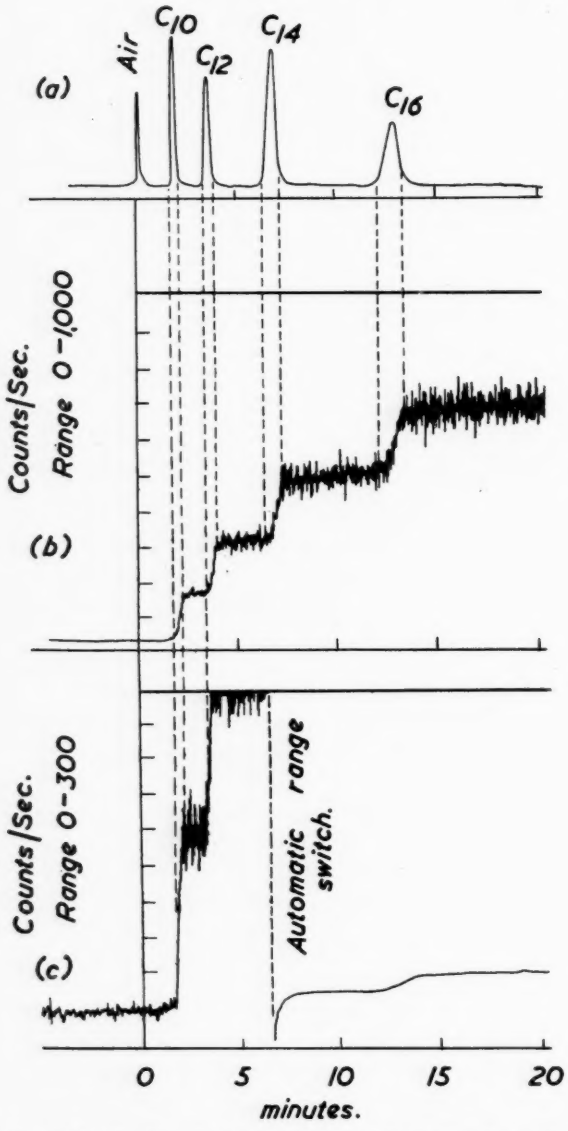


FIG. 6. Gas-liquid radiochromatogram obtained from the analysis of the methyl esters of four C^{14}-labeled fatty acids on ethylene glycol-polyadipate ester column at 197°C. (*a*) Analytical record; (*b*) and (*c*) simultaneous records of radioactivity made at two levels of sensitivity. Total load 1.25 mg. of esters containing 1362 disintegrations/sec. Analytical record taken with one-tenth the normal sensitivity of the gas density balance. See also text.

PERFORMANCE OF THE COUNTER AND SOME OF ITS APPLICATIONS

We considered that the instrument would fulfill its functions satisfactorily if (*a*) the radioactive vapors elicit the response of the scintillation counter within a few seconds after the beginning of the response of the chromatographic analyzer; if (*b*) the width of the record of the radioactive band (integral shaped record) is identical with the width of the analytical (differential) chromatographic record; if (*c*) radioactive peaks following one another very quickly are clearly distinguished; and if (*d*) the response of the counter at all levels of radioactivity, within the ranges of the ratemeter (up to 30,000 pulses per second), is proportional to the radioactivity introduced.

Some of these demands of performance may be best illustrated by records taken during the analysis of a standard mixture of the methyl esters of four C^{14}-labeled fatty acids (C_{10}, C_{12}, C_{14}, and C_{16}) on two columns and in different quantities. The first record (Figs. 6*a*, *b*, *c*) was taken during chromatography of the four acids on a column with a polyester stationary phase (ethyleneglycol adipate) at 197°C and with a total gas flow of 100 ml. per minute (50 ml. per minute through sample column). Under these conditions of very fast analysis, the first fraction (methyl decanoate) required only about 25 seconds to pass through the gas-density balance. It can be seen that the record of radioactivity for each methyl ester is a well-defined step and the time taken for the development of each step corresponds exactly to the time required for the passing of each methyl ester through the density balance. No delay is discernible on the records between the beginning of the analytical record and the beginning of the corresponding radioactive record. By observation of the progress of recording and by timing with a stop watch, however, we found that in every instance the beginning of the radioactive record is delayed after the beginning of the record from the gas-density balance by exactly 10 seconds. This delay is no doubt

due to the time required for the vapors to leave the density balance and for the mixing of the vapors in the scintillator. The analysis shown in Figure 6 is considered to be a severe test of the performance of the instrument on account of the high C^{14}-content of the specimen; the load on the column was so large (1.25 mg. of the esters) that the output from the gas-density balance had to be recorded with one-tenth the usual sensitivity and the total radioactivity contained in the four acids was 1362 disintegrations per second. From this and many other analyses it appears that the resolving power of the scintillation counter is as good as the resolving power of the columns used in chromatography. Figure 6*c* also illustrates the operation of the automatic switch described earlier: on the 3×10^2 range the limit of the range was reached after the appearance of methyldodecanoate and the automatic switch changed this range to 3×10^3 in the middle of the recording of the radioactivity of methyl tetradecanoate.

Another analysis carried out on a slow column with Apiezon-L as stationary phase is shown in Figures 7*a*, *b*, and *c*. As in Figure 6, record *a* represents the analytical chromatographic record from the response of the gas-density balance, and graphs *b* and *c* show the parallel measurements of the radioactivity of the methyl esters at two levels of sensitivity. In this analysis the same mixture of C^{14}-labeled methyl esters was used as in the analysis shown in Figure 6, except that only 430 μg. of the methyl esters were applied to the column. The four methyl esters, C_{10}, C_{12}, C_{14}, and C_{16} were contained in a mixture in the proportions of 1:1:2:2 (w/w), but the specific activities of the C_{14} and C_{16} esters were only about one-half of those of C_{10} and C_{12}. The total radioactivity contained in the 430 μg. of the applied specimen was 466 disintegrations per second, which, when counted on a planchette at negligible thickness with an end-window Geiger-Müller counter (G.E.C. Research Laboratories, Type EHM2, window thickness 1.2 mg. per cm.^{2}), gave 1680 cpm. The total number of counts found in the four methyl esters by the scintillation counting of the vapors was 235 cps., giving a counting efficiency of 50 per cent. From the slower chromatographic analysis on the Apiezon-L column the correct "integral" shape of the tracings of radioactive peaks may be more readily appreciated than from the record shown in Figure 6. It can be seen that the half-value of each step on the radioactive record corresponds very closely to the peaks on the analytical record and thus the radioactivity may be assigned to the corresponding methyl ester identified by the analytical record. As the radioactivity of each ester is given by the height of each step in the record, the specific activity of individual fractions may be calculated if the sensitivity of the analytical detection device is known. As this can be determined by appropriate calibrations, the specific activities of the various fractions may be deduced. For example, the area under the curve representing methyl tetradecanoate corresponds to the response of our gas-density balance to 144 μg. of the ester which gave 66 cps. in the scintillation counter. Thus the specific activity of the methyl tetradecanoate was 458 cps. per mg. ester (or 916 disintegrations per second per mg. ester).

A comparison of the records shown in Figures 6 and 7 indicates also that the response of the counter is strictly proportional to the amount of radioactivity applied to the chromatographic column. In the analysis shown in Figure 6, 1.25 mg. of the esters were used; these gave a total count of 680 cps. in the four fractions. In the second analysis (Fig. 7) 430 μg. of the same esters gave 235 cps., in excellent agreement with the expected figure.

The instrument can also be used as the only chromatographic detection device for specimens containing radioactive substances in so low concentrations that they could not be detected with a gas-density balance nor even with the far more sensitive ionization-chamber detector of Lovelock *et al.* (4). We have encountered such an instance in recognizing, entirely by their radioactivity, a new series of unsaturated branched-chain acids and other volatile substances synthesized enzymically from C^{14}-labeled mevalonic

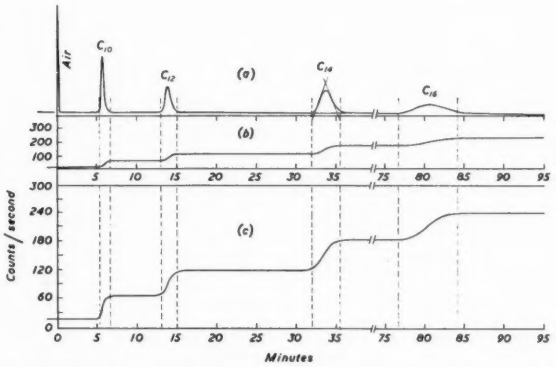


FIG. 7. Gas-liquid radiochromatographic analysis of the methyl esters of C^{14}-labeled fatty acids on Apiezon-L column at 197°C. Total load 430μg. of esters containing 466 disintegrations/sec. (*a*) Analytical record; (*b*) and (*c*) records of radioactivity taken at two levels of sensitivity.

acid (to be published). In Figure 8 is reproduced a composite tracing of a record obtained from the analysis of 5 μg. of methyl esters prepared from a lipid extract of *Chlorella vulgaris* grown from a small inoculum for 3 weeks under photosynthetic conditions in the presence of $C^{14}O_2$ of high specific activity.[3] By the use of the ten times more sensitive range of the counting device, an entirely satisfactory analysis could be carried out with a total of 0.5 μg. of the esters (applied to the chromatographic column in toluene). By the use of markers, introduced together with the C^{14}-labeled esters, most of the radioactive components could be assigned—according to their retention volumes—to well-recognized fatty acid esters, although these had not elicited any response from the gas-density balance.

It should be also pointed out that the slight pressure (approximately 10 cm. of toluene) under which the gas-density meter operates when the counter is attached to it, and the fluctuations of this pressure, owing to the bubbling of gas through the scintillator liquid, have no noticeable effect on the performance of the density meter provided it is correctly balanced.

The day-to-day behavior of the scintillation counter is very constant and analyses of specimens may be reproduced accurately even several months apart.

There is a limitation of the sensitivity of the instrument in that, with the narrow charts (3 in.) used in the recording, radioactive components may be recognized only if the total radioactivity in the component is not less than 2 per cent of the range selected on the ratemeter. Thus, if one selects the 100× range for recording, then 2 counts/second above background—or above the previous level of counting—is readily discernible, but on the 1000× range, 20 cps. are needed to show up as a distinct change in the level of counting. Of course the simultaneous recording at the 10×10^n and 3×10^n ranges increases the sensitivity, so that in the two examples given above, 0.6 and 6 cps., respectively, above previous counting levels may be recognized. After some experience has been gained with the instrument, a change in the counting rate of not more than 1 per cent of the full-scale range may be appreciated. The above limitations are, however, only technical in nature as by the use of larger recorders with a full-scale deflection of 10 inches the sensitivity of the instrument could be improved. Likewise, by employing two photomultiplier tubes on opposite sides of the counting chamber, and connected to a coincidence circuit, a gain in counting sensitivity with lower background count could be achieved although the size and cost of the counter would increase proportionately.

No quenching of the scintillations has been observed by the vapors leaving the chromatographic columns, nor by the small amounts of the stationary phase (Apiezon-L or ethyleneglycol adipate polyester) stripped off the columns. Even after the counting of very active samples (in the region of 10,000 cps.), the background counting rate returns to normal after rinsing out the instrument three times with 25 ml. of clean toluene. This rinsing is usually done through the filling manifold (Fig. 5): the used scintillator is withdrawn into syringe S_1 and ejected into the refuse bottle (R_3), then toluene is drawn up from the reservoir R_2 and injected into the counter as described for filling with the scintillator. Usually 30 to 60 seconds are allowed for the circulation of each washing in the counter for thorough cleaning.

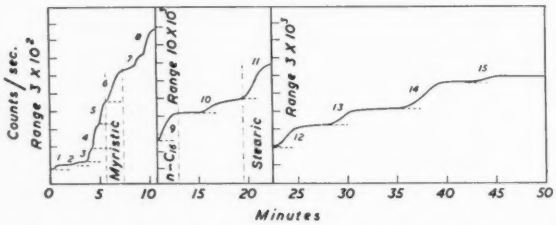


FIG. 8. Gas-liquid radiochromatogram of the methyl esters of fatty acids obtained from *Chlorella vulgaris* grown in the presence of $C^{14}O_2$ of high specific activity. Stationary phase ethylene glycol-adipate polyester; temp.: 197°C. Total load 5 μg. Chromatographic fractions 1, 3, 4, 5, 7, 8, and 10 not definitely identifiable; they are probably various branched-chain acids and/or acids with odd numbers of carbon atoms, e.g., fraction 10 is probably a saturated C_{17} acid. The following fractions could be identified: 2, dodecanoate; 6, tetradecanoate (myristate); 9, palmitate; 11, stearate; 12, oleate; 13, linoleate; 14, eicosanoate; 15, eicosa-mono-enoate. No further component appeared beyond 15.

During an analysis, when several radioactive components have already accumulated in the counter, it is sometimes desirable to change the scintillator in order to return to the more sensitive ranges of the counter; this can be done during a suitable interval between chromatographic fractions as the operation requires only about 1 minute. Washing with clean toluene is usually omitted in such an instance; the height of the next radioactive peak is simply taken from the counting level obtained after the change of the scintillator. This counting level is usually 5 to 10 per cent of that observed before the changing of the scintillator.

A large number of biosynthetic specimens have already been studied by our instrument and it has been found that the most useful ranges for recording were

[3] We are indebted to Dr. J. Catch of the Radiochemical Centre, Amersham, Bucks., for the gift of the lipid extract.

provided by the 10×10^2 and 3×10^2 scales as in the record shown in Figure 8. So far we had to use the most sensitive (100:30) scales very rarely. Of course it is an advantage in arriving at the appropriate selection of ranges for the recording if one knew in advance the approximate number of counts put on the chromatographic columns; this is most conveniently determined by counting an aliquot of the specimen.

Although we have used the instrument so far only for counting C^{14}, there is no reason why it should not be equally suitable for counting tritium or any other radioactive isotope contained in an organic vapor. The instrument has been calibrated with a standard sample of tritio-hexadecane and the efficiency of counting was 20 per cent when electronic noise was 3 cps. and the background counting rate 12 cps.

We are indebted to Messrs. E. K. Cole, Ltd., Southend-on-Sea, Essex, England, for loan of equipment during the development of the counter.

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Qualitative microanalysis and estimation of sphingolipid bases

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SUMMARY

A method is described for the identification and determination of sphingolipid long-chain bases in various animal and plant lipids. Sphingosine and related bases, isolated as a mixture from acid hydrolysates of sphingolipids, are oxidized by sodium metaperiodate and the fatty aldehyde reaction products are isolated and analyzed by gas-liquid partition chromatography. The records thus obtained reflect the composition of the sphingolipid base fraction. A preliminary survey of various tissues is reported and the types of long-chain base found for each tissue are given. Evidence is presented for the presence of a new long-chain base associated with the sphingomyelin fraction of human plasma lipids.

The standard method for the determination of sphingosine and related bases which occur in certain phospholipids, in cerebrosides, and in complex glycolipids has been that of McKibbin and Taylor (1). The method involves a nitrogen determination (micro-Kjeldahl) of a chloroform extract of long-chain base (LCB) hydrochloride and is dependent upon the chloroform insolubility of interfering nitrogenous base hydrochlorides. More recently, Sakagami has described a procedure in which fatty aldehydes, derived from sphingolipid bases by lead tetraacetate oxidation, are determined with fuchsin-sulfurous acid (2). The general reaction for the lead tetraacetate or sodium periodate oxidation of LCB is:

$$R{-}\underset{\underset{OH}{|}}{CH}{-}\underset{\underset{NH_2}{|}}{CH}{-}CH_2OH \xrightarrow[\text{or } Pb(OCOCH_3)_4]{NaIO_4} RCHO + HCOOH + NH_3 + HCHO$$

A preliminary report by Robins and Lowry (3) described a micromethod in which sphingomyelin and total sphingolipids were determined by colorimetric procedures.

These methods provide a quantitative assay for total LCB in lipid hydrolysates. They cannot be used, however, for the qualitative analysis of the sphingolipid bases, which generally occur as mixtures. Sphingosine is the major LCB of mammalian nerve tissue but dihydrosphingosine has also been isolated from this source (4). In addition, phytosphingosine has been reported as a constituent of plant lipids (5) and related plant bases have been described (6, 7). The known sphingolipid bases are listed in Table 1. References to the original structural studies may be found in a recent review by Carter (8).

The lack of a method for distinguishing these bases on a microscale has been a major hindrance to the study of sphingolipid metabolism. The present procedure has been developed as a microassay for the identification and determination of the sphingolipid bases in a mixture. A preliminary description of this work has been reported (9).

METHODS

Materials. Samples of triacetylsphingosine, triacetyldihydrosphingosine and 3-O-methylsphingosine were gifts from Dr. H. E. Carter. The soybean phosphatides were obtained as a gift from the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois. A sample of crude beef spinal cord cerebrosides was obtained from Dr. R. O. Brady. The aldehydes used as standards were obtained from K & K Laboratories, Long Island City, New York, and Aldrich Chemical Company, Milwaukee, Wisconsin. Samples of plasma from patients with Niemann-Pick disease or atherosclerosis were obtained through Dr. D. Fredrickson.

Isolation of Lipids. Total human plasma lipid frac-

tions were obtained by direct extraction of 100 ml. of plasma with 20 volumes of chloroform-methanol (2:1)[1] or alternatively by extraction of lyophilized plasma twice with 5 volumes of chloroform-methanol-water (12:6:1) in a Waring blendor. The white protein fluff in each case was removed by filtration and the extract was washed with one-tenth volume of water and allowed to equilibrate overnight. The bottom layer was concentrated under reduced pressure and the residue dried *in vacuo*.

A commercial powdered yeast (180 g.) was extracted for 10 minutes with 630 ml. of boiling chloroform-methanol-water (10:10:1) and the residue after filtration was re-extracted with 330 ml. of boiling chloroform-methanol-water (10:20:3). The combined extracts were concentrated to dryness *in vacuo* and the residue was dissolved in a small volume of chloroform-methanol (2:1), filtered and reconcentrated *in vacuo*.

Brain and intestinal mucosa were extracted with 20 volumes of chloroform-methanol (2:1) and the extracts were washed with water according to the procedure described by Folch *et al.* (10). The washed extracts were then concentrated to dryness *in vacuo*.

TABLE 1. SPHINGOLIPID BASES

Base	Structure	Source
Sphingosine	$CH_3(CH_2)_{12}CH{=}CHCH(OH){-}CH(NH_2){-}CH_2OH$	Animal
Dihydrosphingosine	$CH_3(CH_2)_{14}CH(OH){-}CH(NH_2){-}CH_2OH$	Animal
Phytosphingosine	$CH_3(CH_2)_{13}CH(OH){-}CH(OH){-}CH(NH_2){-}CH_2OH$	Plant
Dehydrophytosphingosine	$CH_3(CH_2)_xCH{=}CH(CH_2)_{11-x}CH(OH){-}CH(OH){-}CH(NH_2){-}CH_2OH$	Plant
C_{20}-Phytosphingosine	$CH_3(CH_2)_{15}CH(OH){-}CH(OH){-}CH(NH_2){-}CH_2OH$	Plant

Silicic Acid Chromatography. Mallinckrodt No. 2847 silicic acid was prepared for chromatography in the following manner: The silicic acid was washed with 3N hydrochloric acid and rinsed with water until the acid was completely removed. The wet silicic acid was collected on a Buchner funnel and washed several times with alcohol and twice with acetone. The product was oven-dried and sieved. For these studies, 150 to 200 mesh silicic acid was found to be satisfactory. A slurry of 20 g. of silicic acid in about 100 ml. of chloroform-methanol (4:1) was poured into a column (1.8 cm. by 23 cm.) and allowed to settle by gravity. After washing with 50 ml. of the same solvent mixture, the column was washed with chloroform until nearly transparent. The packed height was about 18 cm. and the average holdup volume was 35 ml.

Total lipid samples were fractionated into two classes, neutral and polar lipids, by placing up to 600 mg. of lipid on the column in a small volume of chloroform. Neutral lipids were eluted with 250 ml. of chloroform and polar lipids were eluted with 400 ml. of methanol (11).

Chromatographic separations of the phosphatides were made using a modification (12) of the method described by Lea and Rhodes (13). The column was eluted with 250 ml. of chloroform, followed by chloroform-methanol mixtures as follows: 170 ml. of 9:1, 300 ml. of 4:1, 370 ml. of 1:1, and 150 ml. of 1:4. Fractions (10 ml.) were collected automatically and peaks were located by fraction weights. The chloroform-methanol (1:4) fractions, which contained all of the sphingomyelin,[2] were used for sphingolipid assay. No attempt was made to assay for cerebrosides which were eluted in earlier fractions.

Lipid Hydrolysis and Isolation of the Long-chain Base Fraction. A modification of the procedure of Carter *et al.* (4) was used. The lipid sample was hydrolyzed with methanol-2N hydrochloric acid under reflux for 5 to 6 hours (80 ml. methanol and 16 ml. conc. hydrochloric acid; 8 to 10 mg. lipid per ml.). After cooling, a few drops of sulfuric acid were added and the solution

[1] All solvent ratios are v/v.

[2] The authors are grateful to Dr. D. Fredrickson for paper chromatographic identification of sphingomyelin in this peak.

was extracted three times with 2 volumes of petroleum ether (boiling range, 30°-60°C) to remove fatty esters. The methanol solution was concentrated *in vacuo* to about one-half its original volume and after chilling in an ice bath, was adjusted to pH 12 to 13 with a concentrated aqueous potassium hydroxide solution. An equal volume of water was added and the bases were extracted three times with 2 volumes of diethyl ether. The combined ether extracts were washed twice with one-tenth volume of water after which anhydrous sodium sulfate was added to dry the ether solution. The ether was evaporated at reduced pressure and the residue was dried *in vacuo*.

The crude LCB fraction was purified by silicic acid chromatography. This step was necessary to remove traces of fatty acid esters which would interfere with the gas chromatographic assay. A solution of crude LCB in 1 to 3 ml. of chloroform was placed on a small column (about 2 g. of silicic acid prepared as previously described) and esters were eluted with 20 ml. of chloroform. The bases were eluted with 30 ml. of chloroform-methanol (1:4) and the eluate was concentrated to dryness *in vacuo*. With LCB samples smaller than 10 mg. it was necessary to use solvent-washed glassware at this step and for all subsequent steps in order to avoid possible contamination. The yield of purified LCB from 100 ml. of human plasma was 3 to 5 mg.

Phytosphingosine and related bases were obtained from soybean phosphatides by the procedure described by Carter *et al.* (14). The crude LCB fraction was purified by silicic acid chromatography.

Periodate Oxidation. A 0.2 M aqueous solution of sodium metaperiodate was prepared immediately before use. The LCB was dissolved in methanol (5 mg. per ml.) in a 12 ml. centrifuge tube and one-fifth volume of the periodate solution was added. The final solution had a pH of 4 to 5 and contained approximately 15 μmole per ml. of substrate and 40 μmole per ml. of periodate. The reaction mixture stood in the dark at room temperature for 60 minutes, during which time a crystalline inorganic precipitate formed as the oxidation proceeded. No precipitate formed with a reaction blank containing no long-chain base. After an hour 2 volumes of methylene chloride and one-half volume of water were added. The mixture was shaken and centrifuged and the aqueous (upper) layer was re-extracted with 2 volumes of methylene chloride. The combined extracts were filtered through Whatman No. 1 filter paper and concentrated to dryness *in vacuo*. Benzene was added and concentration was repeated in order to remove traces of water. The crude reaction products were purified, using a small (2 g.) silicic acid column from which the aldehydes were eluted with 25 ml. of chloroform. A 10 to 20 per cent solution of aldehydes (based on the weight of purified LCB) was prepared by evaporating the chloroform eluate under a stream of nitrogen. The aldehydes were analyzed within an hour of preparation since these compounds are not highly stable. When necessary, the aldehydes can be stored overnight at −20°C in dilute chloroform solution.

Gas Chromatographic Analysis. The analyses were done using a Model 10 Barber-Colman instrument. An 8 foot, 4 mm. internal diameter glass column was packed with 60 to 80 mesh Celite 545, which was impregnated with a two-layer stationary phase. The Celite was treated with dichlorodimethylsilane and the resultant siliconized Celite was coated with a glutaric acid-diethylene glycol polyester.[3] The details of this procedure for preparing gas chromatographic column packings have been published (15). Columns prepared in this manner were very stable at 130°-200°C and provided excellent separations of saturated and unsaturated compounds in the aldehyde and fatty acid ester series.

Analyses were usually run at 180°C with an argon pressure of 25 p.s.i. and a flow rate of about 100 ml. per minute. With these conditions the slope was obtained for a semilogarithmic plot of retention time versus carbon chain length for a homologous series of authentic fatty aldehydes (Fig. 1). Although the retention times for the various aldehydes varied very little from day to day, the standard mixture was analyzed frequently as a check.

For the analysis of a mixture of aldehydes from an LCB fraction 1 μl. of the chloroform solution (equivalent to about 100 μg.) was injected into the column with a microsyringe. The gas chromatographic analysis required 45 minutes.

Compositions of LCB mixtures were calculated on the basis of the area under each aldehyde peak. Areas were determined by the product of the peak height and the width at half height. The area of the peak representing O-methylsphingosine was added to that of the peak representing sphingosine.

Catalytic Hydrogenation of Aldehydes. Hydrogenation was used to distinguish unsaturated aldehydes and determine their chain length. The aldehyde mixture after assay was evaporated to dryness, dissolved in 5 ml. of ethyl alcohol and hydrogenated with 2 mg. of 10 per cent palladium-charcoal catalyst (for 1 to 10 mg. of aldehydes) at 25°C and 1 atm. hydrogen

[3] The polyester, obtained from Dr. S. R. Lipsky, was made by Union Carbide Chemicals Co., New York, N. Y.

for 30 minutes. The mixture was filtered through a solvent-washed Celite pad and the filtrate was evaporated to dryness *in vacuo*. A 10 per cent chloroform solution of the residue was used for gas chromatographic analysis. After hydrogenation, unsaturated aldehyde peaks disappeared and were replaced by peaks representing the corresponding saturated aldehydes (see Fig. 3).

The Use of Authentic Samples of LCB. Dihydrosphingosine, isolated from an acid hydrolysate of pure triacetyldihydrosphingosine, was oxidized with periodate and analyzed as described previously. The product was chromatographically identical with hexadecanal and no other peaks were observed.

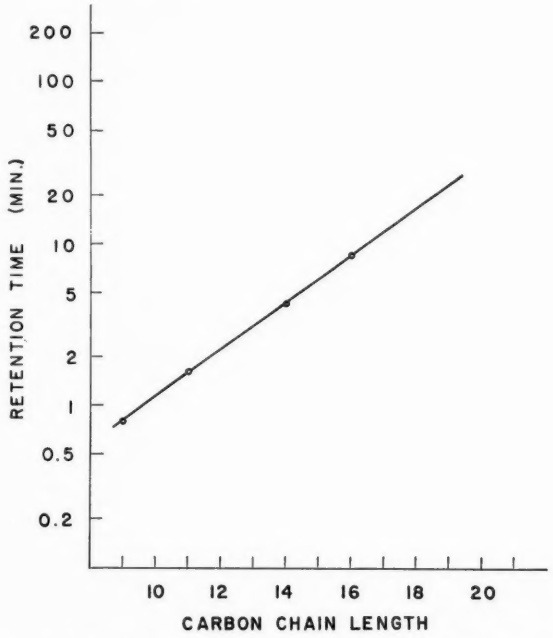

FIG. 1. Retention time of fatty aldehydes as a function of carbon chain length. Conditions of gas chromatography: glutaric acid-diethylene glycol polyester column (8 feet), 180°C, 25 p.s.i. argon.

When a sample of triacetylsphingosine was analyzed by the same procedure the assay showed a major peak (about 80 per cent), representing *trans*-2-hexadecenal, with a retention time nearly identical with that of octadecanal. After hydrogenation of the aldehyde mixture the major peak corresponded to that of hexadecanal. In addition to the major peak two minor peaks were observed which represented about 15 per cent of the total aldehyde fraction (see Fig. 2). These peaks have been identified as periodate products of 3-O-methyl ethers of sphingosine and dihydrosphingosine. The identification was based on comparisons of their retention times with those of aldehydes from authentic base samples. These ether derivatives are products of the methanol-acid hydrolysis (16). It was found that the yield of O-methyl ethers could be minimized by using methanol-hydrochloric acid rather than methanol-sulfuric acid for lipid hydrolysis.

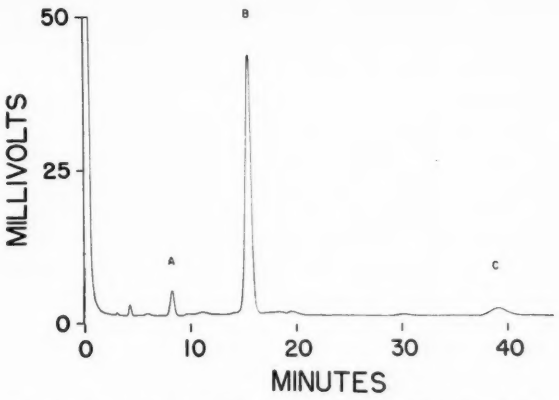

FIG. 2. Gas chromatographic analysis of LCB from bovine brain sphingolipid. Aldehyde peaks represent dihydrosphingosine (*A*), sphingosine (*B*), and O-methylsphingosine (*C*).

It is advisable to analyze solvent concentrates for the presence of high-boiling residues which might interfere with aldehyde analyses. It is usually necessary to use redistilled solvents.

RESULTS

Standard saturated aldehydes were analyzed by gas chromatography in order to determine the relationship between retention time and carbon chain length under a given set of operating conditions. A semilogarithmic plot of these data gave a straight line (Fig. 1) which was used to obtain the retention time for any saturated aldehyde in the homologous series. The conditions for hydrolysis, LCB isolation and periodate oxidation were determined with authentic samples of triacetylsphingosine and triacetyldihydrosphingosine. Periodate oxidation of dihydrosphingosine gave a product which was identical with hexadecanal, as determined by gas chromatography, and periodate oxidation of authentic sphingosine was used to establish the retention time for *trans*-2-hexadecenal.

After this procedure was established and standardized, sphingolipids from a variety of animal and plant tissues were examined. Typical analytical records are reproduced in Figures 2 to 4, which represent, respectively, LCB analyses of bovine brain, human plasma, and soybean phosphatides.

The LCB fraction of bovine brain sphingolipids was composed almost exclusively of sphingosine (94 per cent) and contained only a small amount of dihydrosphingosine.

In contrast to the relatively simple LCB composition of brain tissue, the sphingolipids of human plasma were more complex. As in brain, sphingosine was the predominant base (73 to 82 per cent) but the concentration of dihydrosphingosine was generally higher (5 to 12 per cent). In addition, every human plasma sample contained a new long-chain base to the extent of 10 to 22 per cent. Successive analyses

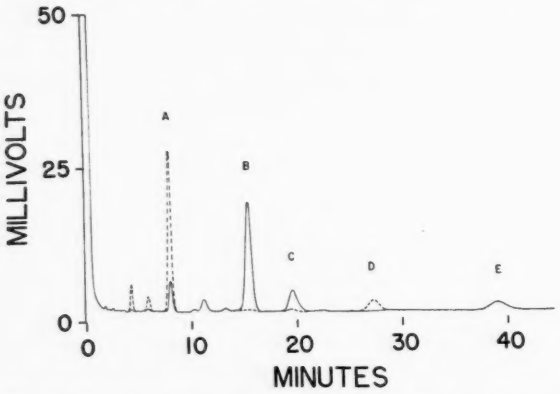

FIG. 3. Gas chromatographic analysis of LCB from human plasma sphingomyelin. Aldehyde peaks (———) represent dihydrosphingosine (*A*), sphingosine (*B*), unidentified plasma LCB (*C*), and O-methylsphingosine (*E*). Record after catalytic hydrogenation (- - -) showing changes in retention time for unsaturated aldehydes (*B* → *A*, *E* → *D*).

were performed on plasma total lipids, crude phosphatides, sphingomyelin fractions from silicic acid chromatography, and sphingomyelin further purified by mild alkaline hydrolysis as described by Sribney and Kennedy (17). In every case sphingosine, dihydrosphingosine, and the unknown base were present in about the same ratio. Catalytic hydrogenation of the aldehydes from sphingomyelin LCB caused both the *trans*-2-hexadecenal and the unknown peak to disappear with a concomitant increase in the hexadecanal peak (Fig. 3).

A sample of soybean phosphatides was hydrolyzed with barium hydroxide according to the procedure of Carter *et al.* (14). Analysis of the LCB fraction showed three peaks (Fig. 4). The two minor peaks were identified as pentadecanal and hexadecanal, representing 10 per cent phytosphingosine and 5 per cent dihydrosphingosine. The major peak (B, Fig. 4) was unsaturated and yielded pentadecanal upon hydrogenation.

Analysis of crude yeast phospholipids, obtained by silicic acid chromatography, showed heptadecanal (38 per cent), pentadecanal (59 per cent), and hexadecanal (3 per cent). The heptadecanal peak confirmed the presence of C_{20} phytosphingosine in yeast as reported by Proštenik and Stanaćev (7).

Sphingomyelin fractions from plasma of patients with Niemann-Pick disease and atherosclerosis were analyzed. In neither case was the result significantly different from that of sphingomyelin from normal plasma.

The results of the various analyses are summarized in Table 2 and lipid fractionation data are given in Table 3.

DISCUSSION

The elucidation of the structures of the sphingolipid long-chain bases was the result of nearly a century of work by numerous investigators since the pioneering studies of Thudichum (18). As a result of these contributions the structures of the known sphingolipid bases have essentially been established (Table 1). There remain several problems of stereochemistry (phytosphingosine) and double bond position (dehydrophytosphingosine) yet to be solved. All these bases have in common the 2-amino and 1,3-dihydroxy groups as shown in Table 1. It is the structure of the remainder of the molecule that is different and characteristic for each base.

These differences afforded the possibility of "fingerprinting" a sphingolipid mixture. An unusual analytical problem was posed by the presence in the different bases of a double bond adjacent to the amino-dihydroxy group (sphingosine), a nonconjugated double bond (dehydrophytosphingosine), an adjacent hy-

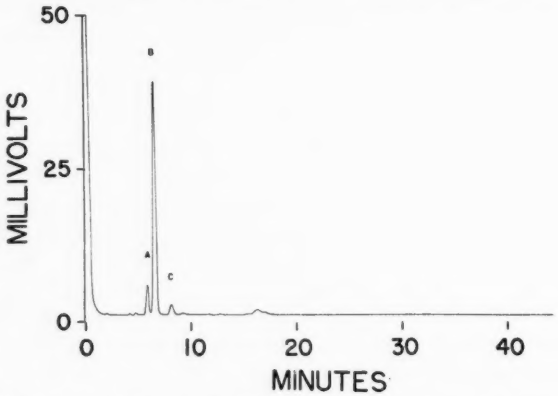

FIG. 4. Gas chromatographic analysis of LCB from soybean phosphatides. Aldehyde peaks represent phytosphingosine (*A*), dehydrophytosphingosine (*B*), and dihydrosphingosine (*C*).

TABLE 2. SPHINGOLIPID BASE COMPOSITION OF VARIOUS LIPIDS

Source	Lipid Fraction	Composition					
		Dihydrosphingosine	Sphingosine *	Unidentified Base	Phytosphingosine	C_{20} Phytosphingosine	Dehydrophytosphingosine
		Percentage	*Percentage*	*Percentage*	*Percentage*	*Percentage*	*Percentage*
Plasma 1	Phosphatides	5	79	16			
Plasma 2	Phosphatides	10	68	22			
Plasma 3	Phosphatides	6	82	12			
Plasma 4	Phosphatides	12	74	14			
		—	—	—			
Average †		8	76	16			
Plasma (atherosclerosis, fem.)	Sphingomyelin	11	77	12			
Plasma (Niemann-Pick disease, fem.)	Sphingomyelin	7	75	18			
Plasma (normal, fem.)	Sphingomyelin	10	73	17			
Bovine spinal cord	Cerebrosides	17	83				
Bovine intestinal mucosa	Phosphatides	3	97				
Bovine brain 1	Phosphatides	6	94				
Bovine brain 2	Phosphatides	7	93				
Yeast	Phosphatides	3			59	38	
Soybean 1	Phosphatides	6			10		84
Soybean 2	Phosphatides	5			10		85

* Figures represent the sum of the sphingosine and 3-O-methylsphingosine peaks.
† Plasmas, obtained from male donors, were A-positive (1-2) and A-negative (3-4) types.

droxyl group (phytosphingosine), and the absence of an additional functional group (dihydrosphingosine). This does not include the additional possibilities of chain length isomers and combinations of functional groups. Gas-liquid partition chromatography was the logical choice as a method for the microdetermination of a mixture of closely related materials. Since the sphingolipid bases were polar and nonvolatile, it was necessary to convert them to volatile derivatives, retaining the structural features characteristic for each base. Periodate oxidation of the sphingolipid bases satisfied these requirements and provided, as well, a simple and quantitative technique. The fatty aldehydes produced by periodate oxidation were easily analyzed by gas chromatography.

In order to separate saturated aldehydes from unsaturated aldehydes of the same chain length, a polar stationary phase was necessary. The glutaric acid-diethylene glycol polyester described by Lipsky *et al.* (19) was excellent in this respect, providing a separation factor [4] of 1.87 for hexadecanal and *trans*-2-hexadecenal.

It was convenient in these analyses to use that amount of tissue from which several milligrams of LCB could be obtained. For example, 100 ml. of human plasma, containing about 10 mg. of mixed sphingomyelins, was sufficient for a complete analysis. In the case of brain, enough sphingolipid was provided by 1 to 2 g. of fresh tissue. It will be possible to extend the method to the analysis of much smaller quantities of tissue. For example, the periodate oxidation may be carried out in a sealed capillary containing 10 μg.

[4] Separation factor is defined as the ratio of retention times for any two components.

of LCB and the entire reaction mixture analyzed, using the capillary injection system described by Bowman and Karmen (20). For this method, 1 to 2 ml. of human plasma or 10 to 20 mg. of brain tissue would be required.

As a result of a partial survey of animal and plant lipids, it is apparent that there are both qualitative and quantitative differences in tissue sphingolipids within a species, as shown in the case of bovine tissue analyses (Table 2). In addition, the sphingolipids from various sources appear to be remarkably different and perhaps characteristic, at least on the basis of the limited data obtained. The interpretation of these data is difficult because of the almost complete lack of knowledge of the functions and intermediary metabolism of this class of lipids.

TABLE 3. FRACTIONATION OF TISSUE LIPIDS

Tissue	Weight	Total Lipid	Crude Phosphatides	LCB Fraction
	g.	*g.*	*mg.*	*mg.*
Plasma 1	8.6 *	0.41	168	2.6
Plasma 2	8.2	0.40	141	3.4
Plasma 3	9.5	0.49	185	2.3
Plasma 4	8.6	0.44	173	<1.0
Plasma (normal, fem.)	8.9	0.61	29 †	1.7
Plasma (atherosclerosis, fem.)	4.0	0.56	28 †	1.3
Plasma (Niemann-Pick disease, fem.)	— ‡	0.57	21 †	8.4 §
Bovine spinal cord			594	85.0 §
Bovine intestinal mucosa	70.0 ‖	1.0	635	<1.0
Bovine brain 1	20.0	1.39	934	40.2
Bovine brain 2	20.0	1.34	1158	48.8
Yeast		14.0	460	4.0
Soybean			236	5.5

* In the plasma experiments, weights represent 100 ml. lyophilized plasma.

† Figures represent the sphingomyelin fractions obtained by silicic acid chromatography.

‡ In this case 90 ml. of plasma were extracted directly.

§ Crude fraction before silicic acid chromatography.

‖ Fresh tissue weights in mucosa and brain experiments.

In the case of plant tissue the characteristic phytosphingosine and related bases are easily recognized since periodate oxidation liberates aldehydes containing an odd number of carbon atoms, e.g., phytosphingosine yields pentadecanal. The major component of the sphingolipids from soybean phosphatides was unsaturated and the chain length was determined by hydrogenation of the aldehyde. The results indicate that this base is dehydrophytosphingosine, which has been isolated in Carter's laboratory (6). Small quantities of phytosphingosine and dihydrosphingosine are also present in soybean phosphatides. This finding is of particular interest since dihydrosphingosine has previously been considered to occur only in animal tissue.

Analysis of crude yeast phosphatides confirms the presence of phytosphingosine and the C_{20} isomer of phytosphingosine as reported by Carter *et al.* (5) and Prošteník and Stanaćev (7), respectively. Dihydrosphingosine is again a minor constituent as in the case of soybean. The method has been applied to the analysis of human plasma sphingolipids. As expected, sphingosine is the major base component and smaller amounts of dihydrosphingosine have been found. In addition, a significant proportion of a new long-chain base is present. These three bases occurred in a similar ratio in every plasma which has been examined, including plasma from a patient with Niemann-Pick disease and a patient with atherosclerosis. The evidence obtained so far suggests that the unknown compound may be a dehydrosphingosine, and studies are currently in progress to isolate and identify the intact base.

The development of this method offers a powerful tool for investigating sphingolipid metabolism. A combination of long-chain base and fatty acid analyses, which may easily be obtained using the same gas chromatography column, provides a method for studying two variables within a mixture of sphingolipids. Thus a fresh approach may be made to the lipidoses in which sphingolipid metabolism is altered, such as Niemann-Pick, Gaucher, and Tay-Sachs diseases.

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Quantitative glass paper chromatography: phosphatidyl choline and sphingomyelin*

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SUMMARY

A rapid chromatographic procedure was developed for the separation of sphingomyelin, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and free fatty acids on glass paper coated with sodium silicate. In addition, phosphatidyl choline and sphingomyelin were determined quantitatively by densitometry of the charred chromatogram, which was obtained by spraying the developed chromatogram with sulfuric acid and heating in an oven. The separation of phosphatides on sodium silicate-treated glass paper is more rapid than on silicic acid-impregnated paper, and the former is simpler to prepare. Preliminary application of this quantitative technique to human serum indicates that it may have a wide adaptability for the determination of phospholipids in natural products.

Phospholipids have been separated on cellulose paper impregnated with silicic acid by Lea and Rhodes (1), Witter *et al.* (2), Hack and Ferrans (3), and others using essentially the same technique. Glass fiber paper impregnated with silicic acid has been used by Dieckert and Reiser (4) and Brown *et al.* (5) for separating phospholipids. According to Agranoff *et al.* (6), cellulose and glass paper give similar results for phosphatide separations when impregnated with silicic acid.

Glass paper offers the advantage of increased sensitivity. Its thermal stability permits the use of concentrated sulfuric acid and subsequent charring by heat for detection of most lipids. The detection of as little as 0.2 μg. of lipid material is below the detection limit of most chromogenic reactions. Swartwout *et al.* (7) have shown that the char formed from spraying with sulfuric acid and subsequent heating can be utilized for the quantitative determination of serum cholesterol, and suggested that the method would be useful for the quantitative determination of a wide variety of organic compounds.

It has been found that glass paper coated with a soluble silicate is superior to one coated with silicic acid owing to its simplicity of preparation and also to the ease of reproducing glass papers with the same properties. It has therefore been possible to determine quantitatively phosphatidyl choline and sphingomyelin in pure solutions. Conditions are described that appear to distinguish qualitatively between phosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and free fatty acids. Some preliminary results of the determination of phosphatidyl choline and sphingomyelin in a Bloor's extract of human serum are presented.

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MATERIALS AND METHODS

Treated Glass Paper. Glass fiber filter paper[1] is cut into rectangles of 10 by 12.5 cm. and heated in a furnace at 600°C for 30 minutes to remove residual organic matter. A 2.0 per cent stock solution of sodium silicate is prepared by dilution of 10.5 ml. of Mallinckrodt sodium silicate solution, 40-42° Be (38 per cent), to 200 ml. with distilled water. The glass paper, suspended by a clip, is wetted by dipping in 0.4 per cent sodium silicate solution, which is made fresh for each use by dilution of the 2 per cent stock solution with water. A clean glass rod is passed across the surface of the paper in such a way as to drain off excess fluid; the papers are suspended over a hot plate until dry, then stored in covered enamel trays until used.

[1] X-934-AH, from H. Reeve Angel and Co., Clifton, N.J.

Reference Compounds and Reagents. Phosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine, and a concentrate of phosphatidyl serine were generous gifts of Dr. Donald J. Hanahan. The properties of these compounds as provided by him are as follows: Phosphatidyl choline from bovine plasma: phosphorus, 3.74 per cent; fatty acid to phosphorus ratio, 1.97; nitrogen to phosphorus ratio, 0.97; choline to phosphorus ratio, 0.95. Phosphatidyl ethanolamine from bovine red blood cells: nitrogen bases, 98 per cent ethanolamine; phosphorus, 4.07 per cent; fatty acid to phosphorus ratio, 2.00; nitrogen to phosphorus ratio, 0.98. Sphingomyelin from bovine red blood cells: nitrogen to phosphorus ratio, 1.97; choline to phosphorus ratio, 0.96. Phosphatidyl serine from bovine red blood cells: contains phosphatidyl serine, phosphatidyl inositol, and some phosphatidyl ethanolamine.

Other reference compounds used were a sample of purified phosphatidyl choline, which was a generous gift of the Upjohn Company, phosphatidyl choline and phosphatidyl ethanolamine obtained from egg yolk by silicic acid chromatography (1) in this laboratory, and a mixture of fatty acids obtained by saponification of corn oil.

The following reagents were Baker Analyzed Reagents and all except pyridine were redistilled before use: Pyridine, benzene, chloroform, diethyl ether, and methanol. U.S.P. reagent absolute ethanol was obtained from U.S. Industrial Chemicals Company and was redistilled before use.

Chromatographic Procedure. The general technique has been previously described (7). Standard reference compounds were dissolved in chloroform-methanol, 2:1,[2] in concentrations ranging from 0.005 to 0.015 per cent. Ten μl. of each standard was applied to the chromatogram giving amounts of each lipid ranging from 0.5 to 1.5 μg. per spot. Other lipid extracts were applied in 10 μl. volumes to contain 0.5 to 1.5 μg. of the desired phosphatide. After spotting, the glass papers were allowed to air-dry for a minimum of 8 minutes.

The chromatogram was first developed in benzene, which moved all the neutral lipids with the solvent front and left the phosphatides at the origin. The chromatogram was air-dried for 8 minutes and developed in benzene-pyridine, 1:1, with varying amounts of water. The chromatogram was air-dried approximately 15 minutes until free of pyridine. The benzene sweep and the solvent development with benzene-pyridine-water each required 7 minutes.

[2] All solvent ratios are given as volume ratios.

The dried chromatogram was sprayed with fresh reagent grade concentrated sulfuric acid so that both sides were evenly but not heavily coated. The sprayed chromatogram was hung in an oven at 230°C for 4 minutes. The density of each charred spot was read in the specially constructed densitometer (7).

Spot Tests. Since the sulfuric acid char has a much lower limit of sensitivity than the various color tests, it was often convenient to separate the phosphatides by chromatography and then to concentrate the separated compounds by the following device. Five, ten, or more 10 μl. aliquots of the lipid extract or standard were applied along a line 2 cm. from the bottom of a coated paper, which was then developed in the desired solvents as described above. After drying in air, this paper was developed in the second dimension, this time in a solvent which moves the phosphatides with or near the front. In this manner the amount of material in a given region on the chromatogram could be increased by a factor of five, if five spots were applied, by ten, if ten spots were applied, and so on. The procedures described by Hack and Ferrans (3) were employed for identifying choline with phosphomolybdate, amino compounds with ninhydrin, and plasmalogens with fuchsin-sulfurous acid-mercuric chloride. Choline containing lipids were also identified by the Dragendorff periodobismuthate reagent as described by Brown *et al.* (5).

RESULTS

Qualitative Separation of Phosphatides. The R_f values in Table 1 were obtained by chromatography on glass paper coated with sodium silicate. The R_f value of any given phosphatide can be varied over a wide range by varying the water content of the developing solvent. A chromatographic separation of phosphatidyl choline, sphingomyelin, and phosphatidyl ethanolamine is shown in Figure 1. These standard samples are chromatographically homogeneous except for phosphatidyl ethanolamine, which leaves a small amount of material at the origin. This probably represents decomposition products formed subsequent to the isolation of the compound.

Two-dimensional chromatograms of the phosphatidyl serine concentrate were obtained by developing first in benzene-pyridine-water, 100:100:10, then in benzene-pyridine-water, 100:100:14. From these chromatograms the phosphatidyl ethanolamine content of the phosphatidyl serine concentrate was estimated to be 10 to 20 per cent. Only two other spots were detected; the faster moving component, the major constituent of the mixture, has been designated in

TABLE 1. R_f VALUES OF PHOSPHATIDES AND FATTY ACID ON GLASS PAPER TREATED WITH 0.4 PER CENT SODIUM SILICATE

Lipid	Volume of Water Added to Benzene-Pyridine Solvent 100:100								
	0	2	4	6	8	9	10	11	14
Free fatty acids	0.65	0.60	0.60	0.60	0.70	0.82	0.85	0.85	—
Phosphatidyl choline	0	0	0	0.25	0.50	0.60	0.75	0.80	0.85
Sphingomyelin	0	0	0	0.04	0.25	0.40	0.50	0.65	0.85
Phosphatidyl ethanolamine	0	0	0	0.02	0.10	0.20	0.25	0.50	0.85
Phosphatidyl serine	0	0	0	0	0	0	0	0	0.60

Table 1 as phosphatidyl serine, and the other component is presumably phosphatidyl inositol.

Quantitative Chromatography of Phosphatidyl Choline and Sphingomyelin. The absorbance of the charred spots obtained after spraying the chromatogram with sulfuric acid and heating was read in the densitometer and was found to be directly proportional to the amount of phosphatide applied to the glass paper. The established useful range of concentrations of sphingomyelin and phosphatidyl choline solutions, applied in 10 μl. volumes, in these studies extends from 0.005 to 0.015 per cent for sphingomyelin, and from 0.005 to 0.020 per cent for phosphatidyl choline solutions. Standard curves for phosphatidyl choline and sphingomyelin are shown in Figure 2. It should be pointed out that the background in the photographs of the chromatograms is not dirt or char but only an artifact of the photographic process. The extreme contrast of the photographic process records the nonuniformity of the glass paper as apparent char spots on the paper. This nonuniformity is easily compensated for in the quantitative densitometry (7).

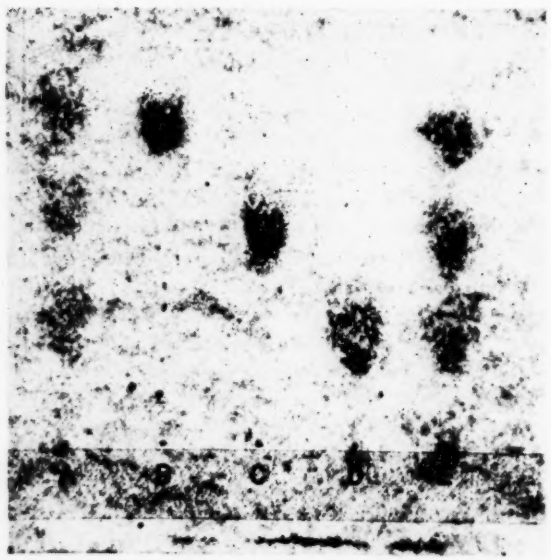

FIG. 1. Photograph of a chromatogram showing the separation of phosphatidyl choline, sphingomyelin, and phosphatidyl ethanolamine. Developing solutions: Benzene followed by benzene-pyridine-water, 100-100-10. Developing time was 7 minutes. A. 0.5 μg. each of phosphatidyl choline, sphingomyelin, and phosphatidyl ethanolamine. B. 1.0 μg. of phosphatidyl choline. C. 1.0 μg. of sphingomyelin. D. 1.0 μg. of phosphatidyl ethanolamine. E. 1.0 μg. each of phosphatidyl choline, sphingomyelin, and phosphatidyl ethanolamine. A contact negative was made on Kodalith film and the print was made on No. 4 contrast projection paper.

Preliminary Results with Human Serum. A Bloor's extract of serum is made by pipetting 1 ml. of serum into a 25 ml. volumetric flask. Six ml. of ethanol-diethyl ether, 3:1, are added in a fine stream and brought just to boiling on a water bath. The solution is cooled, made to volume with ethanol-diethyl ether, and filtered. Suitable aliquots of this extract (1, 2, and 3 ml.) are dried under nitrogen and dissolved in 1 ml. of chloroform-methanol, 3:1. Two 10 μl. aliquots of this solution and similar volumes of three standards are applied to the same chromatogram. This provides a standard curve for each determination. The aliquot whose concentration falls within the desired range is selected for quantitative densitometry.

Two different serum samples having a phospholipid concentration of 280 mg. per 100 ml. (mg. of lipid phosphorus per 100 ml. $\times$ 25) were analyzed by this procedure. The 2 ml. aliquot can be used for the determination of both phosphatidyl choline and sphingomyelin. One sample gave a phosphatidyl choline value of 151 mg. per 100 ml. and a sphingomyelin value of 105 mg. per 100 ml. The other gave a phosphatidyl choline value of 157 mg. per 100 ml. and a sphingomyelin value of 71 mg. per 100 ml. Although the ratios of phosphatidyl choline to sphingomyelin of these two sera are 1.5

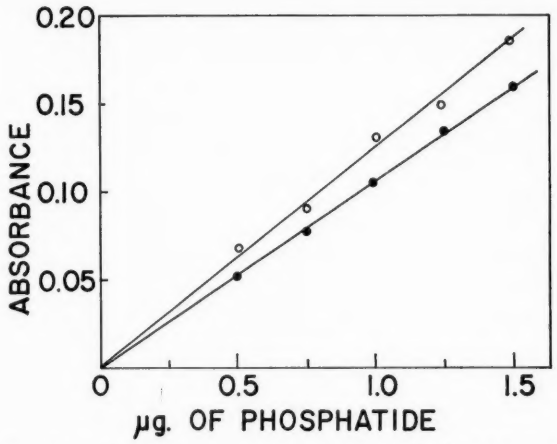

FIG. 2. Standard curves of phosphatidyl choline (●) and sphingomyelin (○). The absorbance of the charred spot is plotted against the amount of phosphatide spotted on the chromatogram.

and 2, ratios as high as 5 have been reported. The chromatogram in Figure 3 shows that both phosphatidyl choline and sphingomyelin are separable and could be determined at ratios as high as 5. The serum sample with a ratio of 2 is included in the figure for comparison. In a single determination on one serum 84 per cent of added phosphatidyl choline and 100 per cent of added sphingomyelin was recovered.

DISCUSSION

Conventional separations of phosphatides on acidic adsorbents, i.e., papers impregnated with silicic acid, are run in acidic or neutral solvents. It is not surprising, therefore, that chromatography of phosphatides on a basic paper, coated with silicate, developed in a basic solvent containing pyridine reverses the order of adsorption. In neutral solvents phosphatides are adsorbed in the same order on silicate paper as in solvents containing pyridine. Although the separation of phosphatidyl ethanolamine and phosphatidyl serine on silicic acid paper is accomplished with time-consuming chromatograms, it was anticipated that they would be easily and rapidly separated on a basic glass fiber paper. The speed of the present method constitutes an important advantage in the handling of the amino phosphatides because of the ease with which these compounds undergo oxidative and hydrolytic decomposition. Chromatographic separation and spot tests of the phosphatidyl serine concentrate indicate that phosphatidyl ethanolamine and phosphatidyl serine are separable by this technique, although further evidence is necessary for absolute proof.

A major problem in the solvent separations of lipids arises from their mutual solubility. Marinetti *et al.* (8) pointed out the desirability of adjusting the composition of the solvent system for phosphatide extracts of different tissues, which is interpreted by us as evidence of lipid mutual solubility effects. The mutual solubility effect is quite pronounced on sodium silicate paper in the benzene-pyridine-water solvent system. However, this difficulty is minimized in the present method by the benzene sweep, a preliminary development of the chromatogram in benzene, which removes all the neutral lipids by sweeping them to the solvent front. This procedure was found to be essential in the analysis of complex lipid mixtures, e.g., serum extract and egg yolk extract, to avoid excessive streaking. It was not found to be necessary for the separation of mixtures of pure phosphatides.

Although this method has the advantage of rapidity, simplicity, reproducibility, and sensitivity, there are limitations which should be pointed out. Satisfac-

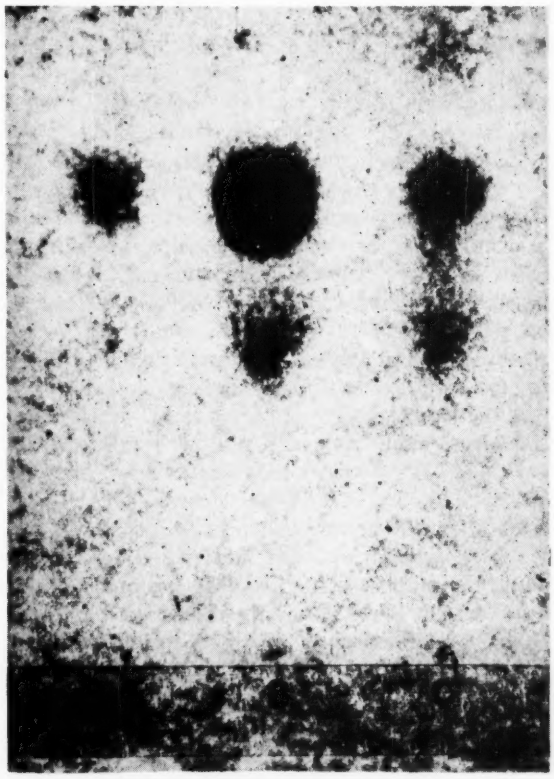

FIG. 3. Photograph of a chromatogram showing the separation of phosphatidyl choline and sphingomyelin at a 5 to 1 ratio and an extract of serum. A. 1.0 μg. of phosphatidyl choline and 0.2 μg. of sphingomyelin. B. 5.0 μg. of phosphatidyl choline and 1.0 μg. of sphingomyelin. C. 10.0 μl. of a 1:12.5 serum extract containing 157 mg./100 ml. of phosphatidyl choline and 71 mg./100 ml. of sphingomyelin.

tory chromatographic separations of phosphatides can be achieved at concentrations as high as 60 mg. of phosphatide per 100 ml. of solution, applied in 10 μl. volumes (cf. *B* in Fig. 3). When the useful range of phosphatide concentrations is maintained between 5 and 15 mg. of individual phosphatide per 100 ml. for quantitative densitometry, it is a simple matter to prepare the indicated dilutions of the lipid mixture and perform the quantitative chromatography separately for each phosphatide on the appropriately diluted extracts. Conversely, when phosphatides are more dilute than 5 mg. per 100 ml., they are concentrated before chromatography. Although this approach increases the number of chromatograms over the requirements of the more conventional cellulose paper chromatography, the time expended is much less.

The chromatographic system herein reported also gives a good separation of free fatty acids. R_f values of fatty acids from corn oil are listed in Table 1. Fatty acids from serum are visible as a spot above phosphatidyl choline in *C* of Figure 3. The feasibility of the determination of free fatty acids in serum by this approach is currently under investigation in this laboratory.

No evidence was obtained to indicate that the chromatographic procedure described hydrolyzes or decomposes the phosphatides. Two-dimensional chromatograms of the pure phosphatides, following the exact procedure as above, were compared with duplicate chromatograms which were developed in identical solvents but from which the solvents were rapidly removed by evaporation in a high vacuum in order to minimize chance of oxidative and hydrolytic decomposition. The chromatograms run under these two sets of conditions were identical by visual inspection.

The identity of the substances from serum which chromatograph the same as pure phosphatides was established by conventional spot tests reagents as follows: phosphatidyl choline and sphingomyelin gave positive choline tests with phosphomolybdate and Dragendorff's periodobismuthate. Phosphatidyl ethanolamine and phosphatidyl serine gave positive ninhydrin reactions. Feulgen's reaction indicated that the major portion of serum plasmalogen ran with phosphatidyl choline. Chromatographic identity of the phosphatides in serum extracts was further established by recovery experiments in which phosphatidyl choline and sphingomyelin appeared in the predicted locations on the chromatogram.

Although the lysophosphatides have not been determined in these solvent systems, from results obtained by others on silicic acid paper, the R_f of lysophosphatidyl choline may be similar to that of sphingomyelin. Because of the basic nature of the paper and the solvent systems, phosphatidyl ethanolamine and phosphatidyl serine have smaller R_f values than phosphatidyl choline and sphingomyelin. Lysophosphatides of phosphatidyl ethanolamine and phosphatidyl serine would be expected to have even smaller R_f values, and therefore not interfere with the determination of phosphatidyl choline and sphingomyelin. The feasibility of applying this method to the determination of all phosphatides in serum and other biological samples is under investigation in this laboratory.

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The microtitration of total fatty acids of serum, with notes on the estimation of triglycerides *

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SUMMARY

A method is described for the determination of total fatty acids of serum by microtitration. The method is designed to permit also the determination of cholesterol and lipid phosphorus, and of triglycerides by difference. One ml. of serum is sufficient for duplicate determinations of total fatty acids, lipid phosphorus, and cholesterol. The essential steps include extraction of serum lipids, saponification, extraction and microtitration of the liberated fatty acids. A possible modification which may be useful in the estimation of triglycerides is described. Recoveries of pure fatty acids and triglycerides were 97 per cent complete. Short chain fatty acids and intermediates of carbohydrate metabolism were not detected by this method.

Fatty acids normally represent about 50 per cent of the total serum lipids. The fatty acids occur chiefly in phospholipids, esters of cholesterol, and in triglycerides, but small moieties also are present as di- and monoglycerides and as free fatty acids bound to albumin. No single lipid constituent accurately represents the status of the other lipids. For clinical as well as research purposes a lipid analysis is not complete unless the three major classes of lipids are measured. The concentration of total fatty acids provides an index of total lipids in terms of their common denominator, and when determined with cholesterol and phospholipids, permits by calculation estimation of triglycerides. The latter are of significance in coronary artery disease, diabetes, and other metabolic disorders as well as in the physiological transport of fat (1 to 4). Since chemical reactions are best expressed as taking place between equivalent weights rather than absolute weights of compounds, the concentration of total fatty acids is most usefully expressed in terms of chemical equivalents rather than absolute weight. A need thus exists for a practical yet accurate method for determining total fatty acids as equivalents, preferably a method sharing as many steps as possible with the chemical procedures necessary for determination of cholesterol and phospholipids.

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Titrimetric determination of total fatty acids after hydrolysis of serum lipids permits direct estimation of total fatty acids as equivalents. With modern micro apparatus 4 ml. of serum seems disadvantageous and excessive for duplicate determinations of cholesterol, phospholipids, and fatty acids measured by the Man and Gildea modification of the Stoddard and Drury method (5).

The present method provides a technique for the microtitrimetic determination of total fatty acids of small amounts of serum. The method is of sufficient simplicity to permit its use as a routine procedure, yet accurate enough for use as a research tool. It was designed to permit also the determination of cholesterol by the method of Abell *et al.* (6) and of phospholipids by the Sperry method (7). One ml. of serum is sufficient for duplicate determinations of these three substances, although less than two-tenths of a ml. of serum is actually used for duplicate titrations of total fatty acids alone.

The lipids are initially extracted by the method of Folch *et al.* (8) with certain modifications. There follows a saponification procedure similar to, but more intensive than, that recommended by Abell *et al.* for total cholesterol (6). Following acidification of the saponified lipids, the liberated fatty acids as well as cholesterol are extracted by shaking with petroleum ether or hexane. The final step is the direct microtitration of the fatty acids in a hexane-alcohol two-phase system similar to that which has been described

by Dole (9) for the titration of "nonesterified fatty acids."

METHODS

Reagents.

Chloroform (reagent grade), redistilled twice weekly.

Methanol (reagent grade), redistilled twice weekly.

Hexane (reagent grade). The Fisher product is used without further purification.

Absolute ethyl alcohol (reagent grade). U.S. Industrial Chemical Company absolute alcohol is used without further purification.

Chloroform-methanol (exactly 2:1 v/v).

Alcoholic potassium hydroxide: 6 cc. of 33 per cent potassium hydroxide are added to 94 cc. of absolute alcohol daily (6).

1 per cent phenolphthalein in 95 per cent alcohol.

1.8 N hydrochloric acid.

Nile blue indicator: 0.02 per cent aqueous Nile blue A solution is washed four or five times with hexane, then diluted 1:10 with absolute alcohol (10).

Standard 0.02 N sodium hydroxide.

Extraction. All determinations are carried out in duplicate. The procedure for a single determination is described. One half ml. of serum (or plasma) is pipetted into a 50 ml. round bottom glass-stoppered centrifuge tube.[1] Exactly 10 ml. of 2:1 chloroform-methanol are added to the serum with constant and vigorous swirling during the addition. The centrifuge tube is then stoppered, sealed with a drop of water, and shaken gently with a back-and-forth motion for 1 minute. The chloroform-methanol-serum mixture is allowed to stand at room temperature with occasional gentle agitation for 1 hour. Ten ml. of distilled water are then added gently down the side of the tube. The tube is stoppered without further shaking and allowed to stand in the refrigerator overnight, or for several days if desired. A reagent blank, included in each run, is prepared by the addition of 10.5 ml. distilled water to 10 ml. chloroform-methanol and treated the same way as the sera throughout. After 15 hours the mixture has separated into a lower chloroform phase and an upper aqueous phase which contains impurities of the original mixture soluble in methanol and water. The precipitated proteins are largely concentrated as a disk between the upper and lower phases.

Most of the lower chloroform phase is removed through a long metal cannula[2] or long hypodermic needle attached to a 10 ml. hypodermic syringe. The cannula is passed carefully through the aqueous phase and the protein precipitate by sliding it down the wall to the bottom of the centrifuge tube. About 5.5 ml. of the 6.67 ml. of clear chloroform extract can be removed and transferred to a test tube from which aliquots are then taken. At this stage it is convenient to remove 1 ml. for lipid phosphorus determination (7) if desired. From the remaining chloroform extract, 4 ml. are measured into a clean 50 ml. glass-stoppered centrifuge tube for determination of cholesterol and total fatty acids.

Saponification. The chloroform is readily evaporated from the unstoppered centrifuge tubes by placing the tubes in a large vacuum desiccator equipped with a sand bath which has been preheated to 50°C. The desiccator is evacuated by a water aspirator pump; 45 minutes is usually sufficient to bring the extracts to dryness. Within a few minutes of the completion of the evaporation (the lipids must not be left dry) 5 ml. of alcoholic potassium hydroxide and 1 drop of phenolphthalein are added to the dried lipid extract in the centrifuge tubes. The tubes are incubated in a water bath at 80°C for 1 hour. Approximately 5 ml. of water are then added to each tube. After 10 more minutes in the water bath, 1 ml. of 1.8 normal aqueous hydrochloric acid is added to the warm hydrolysate and the mixture swirled. This is a little more than enough hydrochloric acid to neutralize the potassium hydroxide and should decolorize the phenolphthalein and precipitate the liberated fatty acids as a fine cloud.

When the mixture has cooled to room temperature, exactly 10 ml. of hexane are added to each tube. The tube is stoppered, sealed with a drop of water, shaken vigorously for 1 minute, and centrifuged for 5 minutes at about 2000 rpm. The clear upper hexane phase then contains virtually all of the liberated fatty acids and the cholesterol now as free cholesterol.

If cholesterol is determined, an appropriate aliquot (5 ml. is satisfactory) may now be pipetted from the upper hexane phase for cholesterol determination by the method of Abell *et al.* (6). Then 3 ml. are pipetted from the remaining hexane phase into a 15 ml. centrifuge tube for determination of total fatty acids. Rubber bulb Propipettes® have been found useful in removing the aliquots from the upper phase without disturbing the lower phase.

Microtitration of Total Fatty Acids. Now 3 ml. of Nile blue indicator are added to the 3 ml. of hexane extract in a 15 cc. centrifuge tube. This two-phase system, an upper hexane phase containing the fatty acids and a lower alcohol phase containing the indicator, is titrated directly by adding 0.02 normal NaOH from a microburette. The 1 ml. Gilmont ultramicroburette

[1] Corning centrifuge tubes #8424 are excellent.

[2] Becton-Dickinson stainless steel laboratory cannula, #B-D 1250NR.

has been found satisfactory. For about 2 minutes before and also during the titration a stream of nitrogen, which has been through a solution of 300 ml. of indicator with 40 ml. of 33 per cent NaOH to remove carbon dioxide, is bubbled through the hexane and indicator through a fine glass tube leading to the bottom of the centrifuge tube. This serves to mix the two phases and to exclude carbon dioxide from the air. The end point is indicated by a change in color from blue to pink.

Calculations. The concentration of total fatty acids in meq. per l. is calculated according to the following formula:

$$\text{Total fatty acids in meq./l.} = (\text{ml. of 0.02 N NaOH} - \text{blank}) \times \frac{1000}{\text{ml. serum extracted}} \times 0.02 \times \frac{\text{total chloroform}}{\text{chloroform aliquot}} \times \frac{\text{total hexane}}{\text{hexane aliquot}}$$

Example: 0.5 ml. of serum was extracted with 10 ml. of 2:1 chloroform methanol. After removal of the methanol, 4 ml. of the 6.67 ml. of chloroform were removed for total fatty acid and cholesterol determination, and the solvent evaporated. After saponification, and addition of 10 ml. of hexane, 3 ml. of the hexane were measured for titration of total fatty acids. The unknown required a titer of 0.0755 ml. of 0.02 N sodium hydroxide; the blank gave a titer of 0.0080 (the blank is almost entirely due to the indicator and is constant from day to day). Using the above formula, it was determined that the concentration of total fatty acids in the serum was 15.0 meq. per l.

$$(0.0755 - 0.0080) \times \frac{1000}{0.5} \times 0.02 \times \frac{6.67}{4} \times \frac{10}{3} = 15.0 \text{ meq./l.}$$

Calculation of Triglycerides. The triglyceride concentration expressed as milliequivalents of triglyceride fatty acids per liter is calculated by subtracting the fatty acids of cholesterol esters and phospholipids from the total fatty acids.

The cholesterol is assumed to be 72 per cent esterified (11, 12). This ratio varies between the narrow limits of 68 to 76 per cent in the sera of normal persons (11). The assumed value of 72 per cent would therefore be within 6 per cent of the actual value. Although small increases in the ratio of esterified cholesterol to total cholesterol are common in diabetic acidosis and essential hyperlipemia, the triglycerides are also increased so that any error incurred by the use of the above formula would be small compared to the total triglycerides (4). In the presence of liver disease or obstructive jaundice, however, the ratio of esterified cholesterol to total cholesterol may be greatly reduced (13) and the free cholesterol should be measured rather than assumed. Unless the serum is obviously icteric, the above formula can be used with impunity.

The phospholipid fatty acids are estimated by assuming that 20 per cent of the phospholipids have one fatty acid molecule per atom of phosphorus (sphingomyelin) and that the remaining 80 per cent have two fatty acid molecules for every phosphorus atom (lecithin and cephalins) (11). The assumption regarding sphingomyelin has been borne out by Phillips' recent report (14) that sphingomyelin constitutes 18 per cent of normal serum phospholipids.

The fatty acids not accounted for as cholesterol esters or phospholipids are assumed to be derived from triglycerides, recognizing that a small fraction of these are present as free fatty acids or as mono- and diglycerides (9, 10, 15). The following formula is used, the derivation of which has been discussed previously (11):

$$\text{Triglyceride fatty acids in meq./l.} = \text{total fatty acids in meq./l.} - (\text{cholesterol fatty acids} + \text{phospholipid fatty acids}),$$

where cholesterol fatty acids in meq./l. =

$$\frac{10 \times 0.72 \times \text{total cholesterol in mg./100 ml.}}{386}$$

and phospholipid fatty acids in meq./l. =

$$\frac{10 \times [(0.80 \times 2) + 0.2] \text{ lipid phosphorus in mg./100 ml.}}{31}$$

RESULTS

Reproducibility. The difference between duplicate titrations was usually less than 0.0020 ml. of 0.02 N sodium hydroxide. In the entire range of total fatty acids tested, the mean difference between duplicate determinations was 0.40 meq. per l. ± 0.37 meq. per l. (or 1.98 ± 1.4 per cent). As practice is gained with the microburette, this error is rarely exceeded.

Recoveries. The completeness of recovery of fatty acids was tested by carrying out the entire procedure on solutions containing known concentrations of pure fatty acids or triglyceride.[3] The material to be tested was dissolved in hexane in known concentrations. An appropriate volume of the hexane solution was meas-

[3] Obtained from The Hormel Foundation, Austin, Minn.

ured into a 50 ml. centrifuge tube and the solvent removed *in vacuo*. To the dried lipid was added 0.5 ml. of water if the material was to be recovered alone, or 0.5 ml. of serum if it was to be recovered from serum. The results were calculated as concentration in water (or serum) and are thus directly comparable with the results of analyses of sera.

The recoveries of palmitic acid and triolein, alone or added to serum, were respectively 97.9 ± 2.6 per cent, and 98 ± 3.7 per cent. Lauric acid was also 97 per cent recovered. The concentrations tested ranged from 8 to 100 meq. per l., a range encompassing all normal and most abnormal sera. In concentrations below 8 meq. per l., recoveries were less complete. Since aliquots of the same size were used for all determinations, the method has applicability over a wide range of concentration without need to adjust the size of aliquots. Recovery of fatty acids with shorter chain length than lauric was not investigated because to date these have not been found in appreciable amounts in serum (16). The possibility that short-chain organic acids might interfere with the method was ruled out by analyzing aqueous solutions of pyruvic, citric, aceto-acetic, succinic, and lactic acids in concentrations between 100 and 200 mg. per 100 ml., higher than are likely to occur in serum. The values obtained were indistinguishable from the reagent blank.

As a further test of the method, fatty acid determinations were made on 60 sera which had been determined in the laboratory of Dr. Evelyn B. Man by the method of Man and Gildea (5). These sera were obtained from both normal persons and patients with a variety of disorders associated with abnormal lipids, including several cases of hypercholesterolemia and essential hyperlipemia. The fatty acid concentrations in these sera ranged from 6 to 100 meq. per l. and the titers of 0.02 N sodium hydroxide ranged from 0.027 to 0.45 ml. The mean difference between methods was 0.80 meq. per l., ± 1.0. When the difference between pairs was expressed as percentage of the total fatty acids, the mean difference was 3.6 ± 3.1 per cent. The positive deviations almost exactly equaled the negative deviations, so that the average algebraic difference was only 0.005 meq. per l.

If the lipid phosphorus is not determined, or is measured directly on serum, the total fatty acids can be measured by adding 5 ml. of the alcoholic potassium hydroxide directly to 0.5 ml. of serum and proceeding with the saponification and titration as described. The serum must be swirled during addition of the alcoholic potassium hydroxide, but further shaking only causes clumping of the protein and should be avoided.

The cholesterol method used here was that of Abell *et al.* (6), except that the serum lipids were extracted and dried prior to saponification, acidified, and extracted with hexane rather than petroleum ether after saponification. The extraction procedure was thought to be desirable prior to saponification in order to provide a common starting point for the determinations of total fatty acids, cholesterol, and phospholipids. Recovery of cholesterol added to serum prior to extraction was complete. In 15 sera analyzed by the method of Abell *et al.*, both with and without prior extraction, the cholesterol concentrations averaged 7 mg. per 100 ml. or 2.6 per cent higher in the extracted sera, although care was taken to treat the standards in exactly the same manner as the unknowns. This discrepancy has not been explained but is less than the error of the method (3 per cent).

A comparison between the cholesterol concentrations determined by the method cited here and by the gravimetric determination (17) from an alcohol-ether extract as conducted in the laboratory of Dr. Evelyn B. Man revealed that the gravimetric method gave somewhat lower values. The absolute amount of the discrepancy increased as the concentration of cholesterol increased; the relative discrepancy remained constant at about 7 to 9 per cent. This difference is small compared to the day-by-day fluctuations of serum cholesterol in a given individual. The lower gravimetric determinations may reflect mechanical errors of this technique, which may be greater now than before, since imported sintered glass funnels formerly used may have retained cholesterol digitonide more completely than the domestic funnels now in use.

Nonphospholipid Fatty Acids. The indirect estimation of triglycerides can be reduced in cumulative errors by eliminating the phospholipids, and thus any possible errors in assuming the fraction of total fatty acids contributed by the phospholipids. Advantage is taken of the absorption of phospholipids by silicic acid (18, 19) in the following adaptation of the method of Eder.[4]

The serum lipids are extracted by adding to 0.5 ml. serum 10 ml. of 2:1 chloroform-methanol, followed by 10 ml. of water, as described in the section on extraction. After standing overnight, about 5.5 ml. of the clear chloroform layer are transferred to a fresh 50 ml. centrifuge tube, with the aid of a syringe and long cannula, care being taken not to transfer any water with the chloroform. Approximately 0.5 g. of silicic acid[5] (¼ level teaspoon household measure is

[4] H. A. Eder. Unpublished method.

[5] Silicic acid (Mallinckrodt) 100 mesh, analytical reagent, "activated" by heating in an oven at 105°C for 24 hours.

convenient and measures about 0.44 g. silicic acid) is added to the chloroform extract through a funnel, with gentle agitation during the addition. The glass stopper is promptly inserted and after standing 15 minutes, with frequent brief, gentle agitation, the tube is centrifuged for 10 minutes. Exactly 4 ml. of the clear chloroform supernatant is pipetted into a fresh 50 ml. centrifuge tube. The subsequent evaporation, saponification, acidification, extraction with hexane, and measurement of aliquots for cholesterol and total fatty acid determination, are identical with the procedures described in the section on methods, except that 50 minutes is sufficient for saponification. The fatty acid titer represents the nonphospholipid fatty acids and is composed of triglyceride and cholesterol ester fatty acids. The triglyceride fatty acids are calculated by subtracting the cholesterol fatty acids (see above) from the nonphospholipid fatty acids.

The completeness of recovery of triglycerides by this method was assessed by analyzing solutions of pure triolein, varying in concentration from 8 to 60 meq. per l., and treated exactly as serum, and 96 to 100 per cent of the triolein was recovered. Ten per cent of free fatty acids added (as palmitic) was lost, but the contribution of free fatty acids to total fatty acids is so small that this loss would not have a detectable influence on the concentration of nonphospholipid fatty acids. Treatment with silicic acid did not influence the reagent blank.

Twenty sera of 20 different persons covering a wide range of concentrations of cholesterol, lipid phosphorus, and triglycerides were analyzed both with and without the addition of silicic acid to the chloroform extract. In none of the 20 was there detectable phosphorus in the extract treated with silicic acid. The cholesterol concentration was not changed by treatment with silicic acid. The triglycerides calculated by the two methods are shown in Table 1. There is good agreement between the two methods over the entire range examined. However, there is a distinct tendency for the triglycerides to be higher in the silicic acid method. This suggests either that there is more sphingomyelin present than assumed in the formula for calculating triglycerides, or that phospholipids are incompletely extracted or saponified during the determination of total fatty acids. In either event, the silicic acid method is probably the method of choice for future studies.

TABLE 1. LIPID VALUES OF TWENTY NORMAL AND ABNORMAL SERA: TRIGLYCERIDES CALCULATED IN THE CONVENTIONAL WAY BY DIFFERENCE AND AFTER REMOVAL OF PHOSPHOLIPIDS WITH SILICIC ACID

Serum	Cholesterol	Lipid P	Triglyceride Fatty Acids	
			Conventional	After Silicic Acid
	mg./100 ml.	*mg./100 ml.*	*meq/l.*	*meq./l.*
1	348	11.6	4.1	4.7
2	194	8.4	4.5	3.7
3	410	18.5	53.3	53.2
4	369	11.5	5.6	6.1
5	282	12.0	12.3	14.5
6	286	12.1	6.8	8.2
7	362	12.1	8.7	10.2
8	232	8.8	2.8	3.4
9	310	20.8	52.1	54.0
10	217	10.7	4.6	5.9
11	396	16.1	18.0	20.2
12	210	9.2	6.3	5.3
13	273	11.6	8.6	8.5
14	274	10.5	7.0	9.5
15	147	6.0	2.9	3.3
16	171	8.0	2.8	3.1
17	307	11.5	5.1	5.7
18	231	11.6	5.6	7.2
19	342	15.0	19.1	18.9
20	395	15.1	2.6	3.3

DISCUSSION

The extraction procedure described by Folch *et al.* (8) and adapted to serum by Sperry *et al.* (20) has been simplified in several respects. First, the separate addition of the chloroform and methanol and the heating of the extract (20) were not found necessary to ensure adequate extraction. It was, however, found essential to swirl constantly during, and for a brief period after, the addition of chloroform-methanol to the serum in order to ensure fine dispersion of the protein precipitate in the extraction mixture.

A second modification of the procedure of Folch *et al.* is the elimination of the filtration of the protein precipitate. The cold chloroform is of such great density that the proteins float to the top of the chloroform phase as the methanol diffuses into the upper aqueous phase.

A third modification avoids the removal of the aqueous phase and subsequent washings, as well as the reconstitution of the remaining chloroform with methanol and the necessity of adding solvent to a definite volume before taking aliquots. The modification is based on the assumption that all the chloroform originally added in the chloroform-methanol mixture is quantitatively restored by the removal of the

methanol into the aqueous phase or that traces of chloroform lost to the upper phase are balanced in volume by traces of water and methanol remaining in the lower phase. The measurement of volumes in a long 50 ml. glass-stoppered cylinder graduated in 0.02 ml. divisions, showed that a volume equal to the 20 ml. of chloroform theoretically present in 30 ml. of 2:1 chloroform-methanol mixture was restored by mixing with 30 ml. of water. Under these circumstances the evaporation of chloroform is evidently negligible, for the level of the chloroform water interface remained unchanged after standing at room temperature for 3 months. Changes in laboratory temperature between 20° and 30°C caused negligible volume changes.

Because of the possibility that serum free fatty acids, a small constituent of total fatty acids, might be incompletely recovered (21) unless the aqueous layer were acidified (22), dilute sulfuric acid and 0.5 M sodium acid phosphate buffer (pH = 4.02) were tried in place of water. In neither instance was the recovery of total fatty acids changed in the least.

The saponification is essentially that recommended by Abell *et al.* (6) for hydrolysis of cholesterol esters. Increasing the temperature from 38° to 80°C increased the yield of total fatty acids, presumably by improving the hydrolysis of the phospholipids. Increasing the strength of the alcoholic potassium hydroxide from approximately 2 per cent to 15 per cent or prolonging the time of saponification to 5 hours produced no further increase in total fatty acids. Acidification of the saponification mixture after incubation, necessary for extraction of the liberated fatty acids by hexane, made no difference in the cholesterol determination and did not contribute to the fatty acid reagent blank. The quantitative recovery of the hexane from the hexane-alcohol-water mixture was ascertained by the measurement of appropriate volumes in a graduated cylinder.

The ingenious two-phase titration system used by Dole for titration of free fatty acids (9) was satisfactory, and because the concentration of total fatty acids is so much greater than the concentration of free fatty acids, much smaller amounts of serum were required. The Nile blue indicator recommended by Gordon (10) gave a good end point. The lag in color change, presumably caused by the lag in transfer of fatty acids between phases, was minimized by the use of hexane instead of heptane and by increasing the volume of indicator to equal the volume of hexane solution. The titer of the blank was almost entirely contributed by the indicator, and constituted as much as 20 per cent of the titer of the unknown. Great care was taken, therefore, in the daily titration of the blank and in the accurate addition of the indicator. Lower blanks and better checks were obtained when carbon-dioxide-free nitrogen was allowed to bubble through the test tube for a few minutes prior to titration.[6]

Standardization of the alkali was carried out by weekly titration against dilutions of 0.1 N hydrochloric acid, and the correction factor thus obtained incorporated into each calculation. The burette was filled with sodium hydroxide from a small glass-stoppered bottle filled freshly each day, and flushed out with nitrogen before filling.

The present method has been found readily adaptable to the determination of lipids in lipoprotein fractions as well as in whole serum or plasma. It conserves time since little extra manipulation is required over that necessary for determining lipid phosphorus and cholesterol. When these determinations are also made, the present method compares favorably in both time and accuracy with existing methods, such as the hydroxylamine colorimetric method (22, 23). It might complement the triglyceride method of Van Handel and Zilversmit (24). It gives more chemically specific information than the gravimetric method of total lipid analysis (20), and avoids the assumptions necessary in the colorimetric total lipid method of Bragdon (25). Finally, it gives results closely comparable with those obtained by the method of Man and Gildea (5). Any future work done with the present method can therefore be compared with the studies of Man and Peters and their co-workers and can thus draw upon the wealth of information which has been given to the field of lipid investigation by these authors.

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Positional distribution of saturated and unsaturated fatty acids on egg lecithin*

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SUMMARY

A method is described to determine the position of fatty acids on lecithin. Purified egg lecithin is enzymatically hydrolyzed to the corresponding mixture of α,β-diglycerides with lecithinase D. Myristic acid is incorporated into the α' position of the diglycerides and the resulting triglycerides are hydrolyzed with pancreatic lipase, which specifically cleaves fatty acids from the α and α' positions. Palmitic, myristic, and stearic acids were freed by the pancreatic lipase, proving that the saturated acids are on the α' position, while the unsaturated fatty acids occupy the β position of egg lecithin. Since snake venom lecithinase A removes the unsaturated acids from lecithin, the site of hydrolysis must be at the β position of lecithin.

It has been known for several years that snake venom lecithinase A liberates unsaturated fatty acids from lecithins containing both saturated and unsaturated fatty acids (1). In recent years this observation has been confirmed in more detail by several investigators and the resulting lysolecithin has been shown to contain mostly saturated fatty acids (2 to 5). These observations cannot be explained on the basis that lecithinase A is specific for the hydrolysis of only unsaturated fatty acid ester linkages, since both di-saturated and di-unsaturated lecithins have been shown to be hydrolyzed equally well to yield one mole of fatty acid and the corresponding lysolecithin (6). It was therefore concluded (7) that the action of the enzyme was directed toward one particular ester group, α' or β, in the lecithin molecule.

Hanahan (8) attempted to find the site of attack of the enzyme by determining the position (α' or β) of the free hydroxyl group in lysolecithin. After oxidation of the lysolecithin with permanganate, followed by acid hydrolysis, he isolated phosphoglyceric acid in 65 to 85 per cent yield as the only detectable phosphate ester and concluded that the free hydroxyl group was in the α' position; therefore the lecithinase A specifically hydrolyzed the ester linkage in the α' position.

Long and Penny (9) reached the same conclusion when they found that lysolecithin was oxidized by acid dichromate with an uptake of four equivalents, as expected for a compound containing a primary hydroxyl group. Later Davidson *et al.* (10) isolated an acidic product of the permanganate oxidation of lysolecithin and showed it to be lysolecithinic acid. Gray (11) has recently confirmed this finding. Neither of the latter groups, however, reported any yields for lysolecithinic acid, nor did they look for the presence of other phosphate esters in the products of oxidation.

Marinetti *et al.* (12) have recently repeated Hanahan's oxidation procedure but obtained evidence for the presence of a keto-lysolecithin as well as lysolecithinic acid. After acid hydrolysis of the oxidation mixture, four water-soluble phosphate esters were detected: glycerophosphate, phosphoglyceric acid, phosphorylcholine, and orthophosphate. They concluded that lysolecithin was a mixture of α and β isomers, and that the enzyme hydrolyzes ester linkages at both the α' and β positions. Marinetti *et al.* (13) also found that snake venom lecithinase A would hydrolyze ester linkages from the β position of beef heart plasmalogens. They interpreted this latter finding as further proof for the lack of specificity of lecithinase A.

McArthur and Bennett also repeated the oxidation of snake venom-produced lysolecithin with permanganate and found that 74.5 hours at 37.5°C were necessary for the uptake of 2 atoms of oxygen per mole of lysolecithin. Methylglyoxal was present after acid hydrolysis but they were unable to detect any phos-

* Issued as N.R.C. No. 5379.

phoglyceric acid. They concluded that the lysolecithin must have been predominately the α' acyl lysolecithin.[1]

In view of the conflicting results obtained by permanganate oxidation procedures, it appeared desirable to try an entirely different and independent approach to the problem of the specificity of lecithinase A. Basically, the question of the specificity of lecithinase A, at this stage, amounts to the positional arrangement of the fatty acids in the lecithin used as a substrate. In recent years the determination of the positional arrangement (α or β) of fatty acids in triglycerides has been made possible by the discovery that pancreatic lipase initially catalyzes the hydrolysis of only primary ester linkages (14, 15, 16). Therefore by converting purified egg lecithin to α,β-diglycerides—by the action of lecithinase D (17)—introducing a known fatty acid at the primary hydroxyl group of these diglycerides, subjecting the resulting triglycerides to the action of pancreatic lipase, and analyzing the free acids liberated, it should be possible to determine which fatty acids were originally present on the α' and β positions of lecithins (Fig. 1). Comparison with the fatty acids liberated by lecithinase A should then establish which fatty acid-ester linkages are cleaved by this enzyme (Fig. 2).

The present paper provides new evidence on the position of saturated and unsaturated fatty acid esters in purified egg lecithin.

EXPERIMENTAL

Analytical Methods. Phosphorus was determined by King's method (18), total nitrogen by a micro-Kjeldahl procedure (19), and total choline by Glick's method (20).

Fatty acid analysis was carried out as follows: The lipid sample (150 mg.) was saponified with 10 ml. of aqueous N NaOH by reflux on a steam bath for 3 hours. After cooling, the mixture was acidified with 50 ml. of 5 N HCl. The fatty acids were extracted with ethyl ether and the ethereal solution washed with water, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* at 35°C in a stream of N_2. The residue was dissolved in hot methanol: water (9:1; v/v) and titrated with 0.02N NaOH in 90 per cent aqueous methanol to the o-cresol red end point. After acidification, the free fatty acids were extracted with petroleum ether (B.P. 30-60°C), concentrated in a stream of N_2, dried over P_2O_5 *in vacuo*, and weighed. The acids were then dissolved in ethyl ether, converted to the methyl esters with diazomethane, and analyzed by gas-liquid partition chromatography.

Gas-liquid partition chromatography was carried out on a Podbielniak "chromacon" (Series 9475-3V), with a thermal conductivity detector, which had been

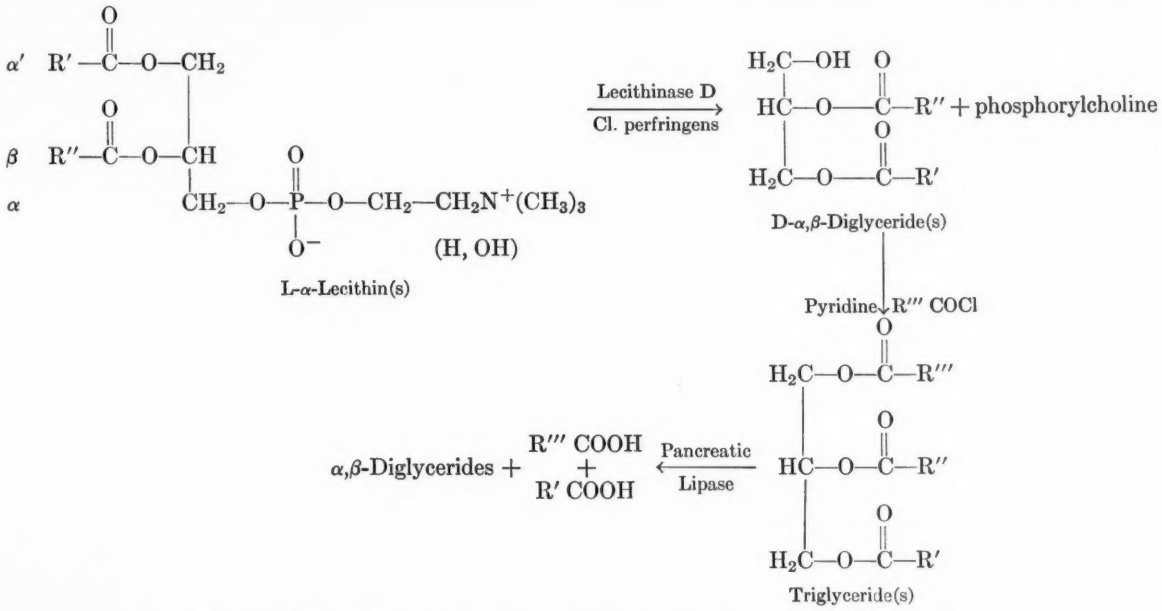

FIG. 1. Reaction scheme for the proof of positional distribution of fatty acids on lecithin.

[1] Communication from Dr. C. S. McArthur and Mr. J. R. Bennett, University of Saskatchewan, Saskatoon, Sask.

modified to take glass columns and to improve the pressure regulation, temperature control, and collection and injection systems. The glass chromatographic columns (6 mm. internal diameter by 8 feet length) were packed with Celite 545 impregnated with a polyester of 1,4-butanediol succinate (7/3; w/w) by the method of Craig.[2] Helium was used as a carrier gas.

No attempts were made to recover all the fatty acid esters from the chromatogram. The amount of each fatty acid ester was determined by measuring the peak area, a factor converting peak area to moles percentage of each of the acids having been previously determined with a known mixture.

Preparation of Egg Lecithin. Egg lecithin was prepared by the method of Rhodes and Lea (21) using alumina and silicic acid columns. Yield of acetone-precipitated lecithin was 9.85 g. per dozen fresh eggs. The final product was found to be chromatographically homogeneous on silicic acid-impregnated paper when stained with phosphomolybdic acid (22). The fatty acid composition of "purified lecithin" is given in Table 1, from which the mean molecular weight was calculated to be 273. On analysis the lecithin gave the following values for: N, 1.82; P, 3.94; C, 64.93; H, 10.74; ester choline, 15.27; mean molecular weight of fatty acids, 276; total fatty acids, 70.3 per cent; N to P molar ratio, 0.98; fatty acid to P molar ratio, 1.99; choline to P molar ratio, 0.99; calculated for $C_{42.5}H_{85}O_9NP$ (m.w. 785): N, 1.78; P, 3.95; C, 65.04; H, 10.87; ester choline, 15.44; mean molecular weight of fatty acids, 273; total fatty acids, 69.5 per cent.

Preparation of Lysolecithin. Egg lecithin (150 mg.), containing 5.95 mg. of P., was dissolved in 100 ml. of diethyl ether and treated with 1 ml. of 0.1 per cent rattlesnake venom (*Crotalus adamanteus*) in 0.005 M $CaCl_2$ solution (23). The solution was allowed to stand overnight at room temperature, the precipitate was removed by centrifugation and washed once with diethyl ether. No phosphorus was present in the ether phase, indicating that the reaction was complete. The free fatty acids in the ether phase and those remaining in the lysolecithin were analyzed by gas-liquid partition chromatography (Table 1 and Fig. 4). The lysolecithin was chromatographically homogeneous on silicic acid-impregnated paper when stained with phosphomolybdic acid (22).

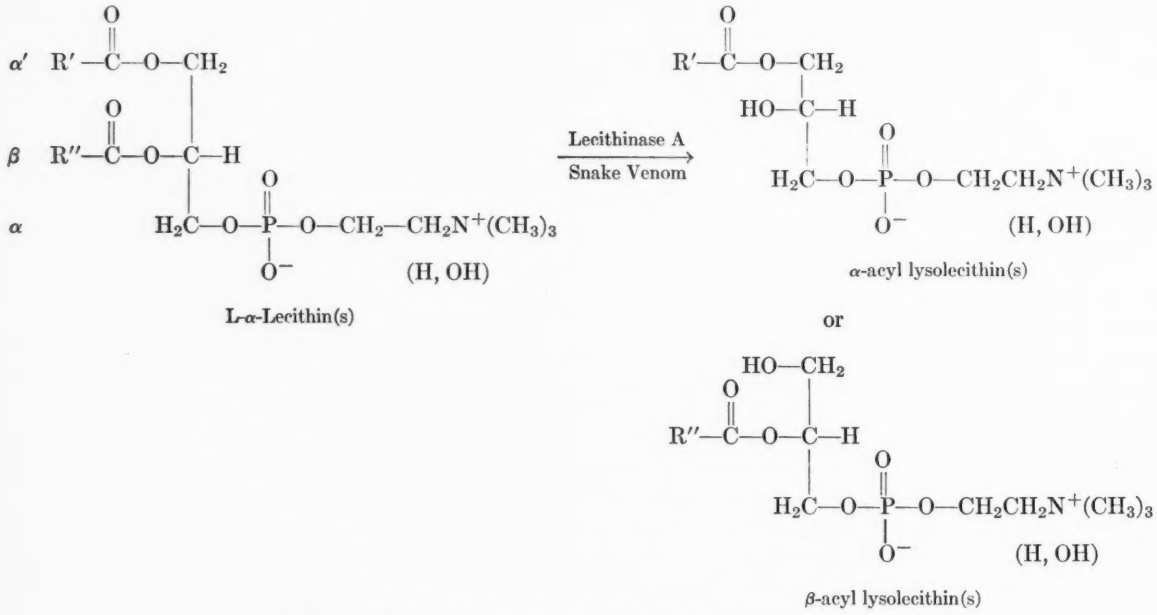

FIG. 2. Reaction scheme for the action of snake venom lecithinase A on lecithin.

Preparation of d-α, β-diglyceride. Purified lecithin was converted to the corresponding α,β-diglycerides by the method of Hanahan and Vercamer (17) using *Cl. perfringens*.[3] From 1 g. of egg lecithin, 748 mg. of

[2] Communication from Dr. B. M. Craig, Prairie Regional Laboratory, National Research Council, Saskatoon, Sask.

[3] Supplied by Dr. D. J. Kushner, Forest Insect Laboratory, Sault Sainte Marie, Ont.

TABLE 1. FATTY ACID COMPOSITION OF VARIOUS FRACTIONS AND COMPOUNDS

Compound	Moles Per Cent Acid				
	Myristic	Palmitic	Stearic	Oleic	Linoleic
1. Egg lecithin	—	35.7	14.9	37.0	12.4
2. Lysolecithin (formed from egg lecithin by lecithinase A)	—	69.7	30.3	—	—
3. Fatty acids (freed from egg lecithin by lecithinase A)	—	—	—	74.8	25.2
4. α,β-diglyceride (formed from egg lecithin by lecithinase D)	—	35.1	14.6	36.8	13.5
5. Triglyceride (formed from α,β-diglyceride by acylation with myristoyl chloride)	32.1	23.9	9.9	25.0	9.1
6. Fatty acids (freed from above triglyceride by pancreatic lipase in 15 minutes)	48.1	36.0	15.9	—	—
6*a*. Fatty acids (freed from above triglyceride by pancreatic lipase in 25 minutes)	40.1	30.2	13.3	12.1	4.3
7. Fatty acids (freed from 2-oleoylpalmitoyl stearin by pancreatic lipase in 15 minutes)	—	48.3	51.7	—	—
7*a*. Fatty acids (freed from 2-oleoylpalmitoyl stearin by pancreatic lipase in 25 minutes)	—	45.2	46.1	8.7	—

a slightly yellow oil (free of phosphorus) was obtained (theory, 765 mg.). The infrared spectrum of the oil was similar to that of a synthetic d-α,β-dipalmitin (24). Total fatty acids were determined by gas-liquid partition chromatography (Table 1).

Preparation of Triglyceride from α,β-diglyceride. The d-α,β-diglycerides (500 mg.) formed from egg lecithins were acylated with myristoyl chloride in the presence of pyridine, as described in a previous publication (16). After isolation of the triglycerides from the acylation mixture, the dried yellow oil weighed 633 mg. (theory, 676 mg.). The oil was dissolved in 3 ml. of benzene and applied to a column of 15 g. of silicic acid (Mallinckrodt, 100 mesh, prepared by the method of Hirsch and Ahrens (25) to remove fine particles). Elution was continued with benzene and 10 ml. fractions were collected until no more lipid was eluted. After removal of the solvent *in vacuo*, in the presence of N_2, 604 mg. of an almost colorless liquid was recovered. The infrared spectrum was similar to that of a synthetic oleoyldipalmitin. No attempts were made to crystallize the triglyceride mixture. Total fatty acids were analyzed by gas-liquid partition chromatography (Table 1).

Hydrolysis of Triglycerides with Pancreatic Lipase. Synthetic 2-oleoylpalmitoylstearin [4] was used as a substrate in order to test the validity of using pancreatic lipase to cleave specifically fatty acids from the primary positions of triglycerides. Two hundred mg. of the synthetic triglyceride and 200 mg. of the triglycerides formed from the α,β-diglycerides were each hydrolyzed for 15 and 25 minutes consecutively, the free fatty acids were isolated as previously described (16) and estimated as methyl esters by gas-liquid partition chromatography. The results are summarized in Table 1 and Figure 3.

RESULTS AND DISCUSSION

Purified egg lecithin contains four fatty acids, palmitic, stearic, oleic, and linoleic in the molar ratios of 1.0, 0.41, 1.04, and 0.35 (Table 1). In addition to these acids, traces of myristic, palmitoleic, a C_{20}, and a C_{22} acid have been observed, but they were disregarded in the present paper.

As has been previously observed by a number of

[4] Supplied by Dr. W. Landmann, U.S. Department of Agriculture, New Orleans 19, La.

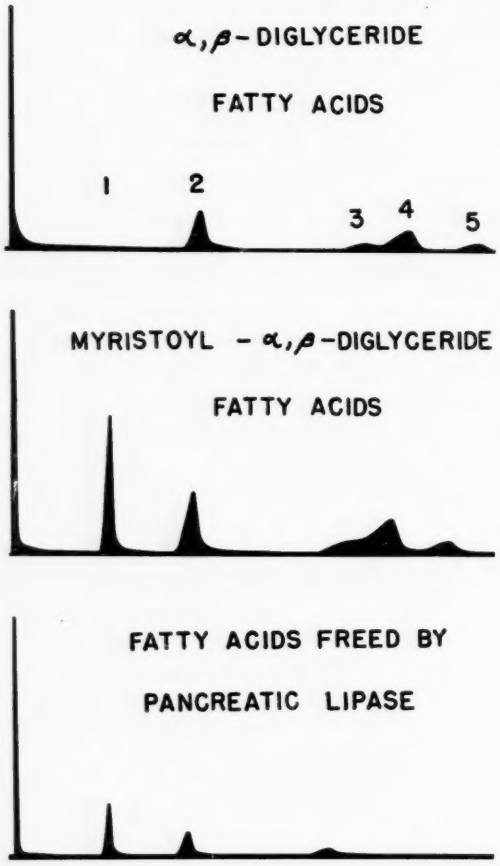

FIG. 3. Separation of methyl esters by gas-liquid partition chromatography, showing the action of pancreatic lipase on myristoyl-α,β-diglyceride. Peak identification: *1*, myristate; *2*, palmitate; *3*, stearate; *4*, oleate; *5*, linoleate.

investigators (2 to 5), snake venom lecithinase A removed only the unsaturated fatty acids, oleic and linoleic, from egg lecithin and left the saturated fatty acids, palmitic and stearic, in the lysolecithin (Table 1 and Fig. 4). There did not appear to be any unsaturated acids remaining in the lysolecithin, although previous workers have observed 0.15 double bonds per mole (4). However, methyl stearate and methyl oleate are not completely separable on the chromatogram and a trace of the latter may have been obscured by the large peak for the C_{18} saturated acid.

When synthetic 2-oleoylpalmitoylstearin was enzymatically hydrolyzed with pancreatic lipase for 15 minutes only palmitic and stearic acids (1:1) were released (Table 1), but if the reaction time was allowed to proceed for 25 minutes, approximately 10 per cent oleic acid appeared. The release of fatty acids originally in the β position of the triglyceride probably indicates hydrolysis of the diglyceride. It is therefore important to standardize the reaction time when using this enzyme to cleave specifically fatty acids from the primary positions of triglycerides.

When the triglycerides formed from lecithin were hydrolyzed by the pancreatic lipase for 15 minutes, only myristic, palmitic, and stearic acids were released (Table 1 and Fig. 3). Furthermore, the mole ratio of myristic acid to palmitic plus stearic acid was very close to 1. This is unequivocal evidence that only saturated fatty acids are present in the α′ position and that the unsaturated fatty acids are in the β position of egg lecithin. Since snake venom lecithinase A removes only unsaturated fatty acids, and, as shown above, these occur on the β position, the lysolecithin must be the α′ acyl ester. This finding is contrary to the conclusions of Hanahan (8) and of Long and Penny (9), but the possibilities of acyl migration, occurring during enzymatic hydrolysis of lecithin, or the oxidation of lysolecithin, or both, were not elim-

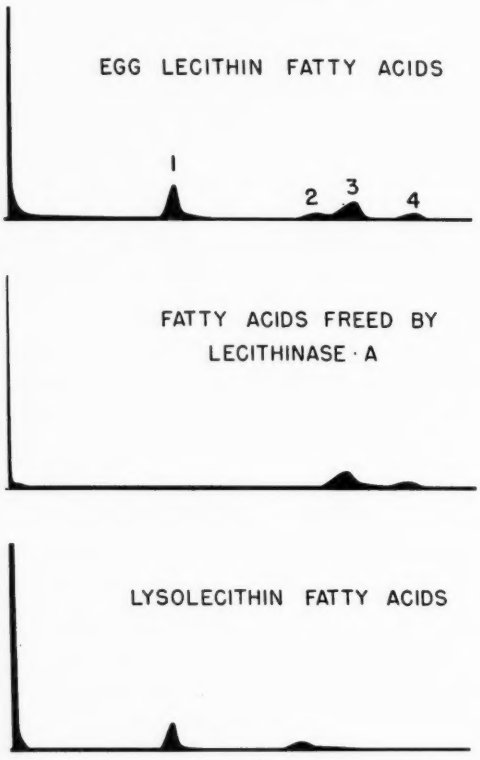

FIG. 4. Separation of methyl esters by gas-liquid partition chromatography, showing the action of lecithinase A on lecithin. Peak identification: *1*, palmitate; *2*, stearate; *3*, oleate; *4*, linoleate.

inated in their experiments. The present method, on the other hand, is based on reactions in which acyl migration is highly unlikely. The action of lecithinase D on lecithin has been shown to produce a d-α,β-diglyceride without racemization (17, 26), and acylation in the presence of pyridine does not cause acyl migration (27).

Mattson and Lutton (28), as well as Savary and Desnuelle (29), have recently found that there is some degree of specificity in the arrangement of fatty acids in glycerides. They found that most glycerides have unsaturated fatty acids in the β position. The finding that egg lecithin also has unsaturated fatty acids in the β position suggests that there may be a direct relation between the biosynthesis of triglycerides and phospholipids.

The author is deeply indebted to Mr. A. G. McInnes and Dr. M. Kates for their valuable advice. Sincere thanks are also expressed to Mr. F. Cooper for all the gas-liquid partition chromatographic analyses. The author expresses his gratitude to Mr. A. Castagne for C, H, and N analyses.

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The isolation and identification of lysolecithin from lipid extracts of normal human serum *

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SUMMARY

Chromatography on silicic acid was employed for the separation of phospholipid components of normal human serum. These components were identified as cephalins, lecithin, sphingomyelin, and lysolecithin by paper chromatography, chemical analyses, and infrared spectra. The quantities of the phospholipid component were determined.

A recent paper by Hirsch and Ahrens (1) has described the separation of the major classes of lipids in human serum by silicic acid chromatography. The major phospholipid constituents of serum were reported to be cephalins, lecithins, and sphingomyelin eluted from the column in that order.[1] Phillips (2, 3) has isolated a fourth phospholipid component of serum and presented evidence for its identification as lysolecithin, using the silicic acid chromatography technique of Lea *et al.* (4).

The present study offers conclusive confirmation of the presence of lysolecithin and identification of the major phospholipid components of normal human serum separated by the silicic acid chromatographic technique of Hirsch and Ahrens (1).

METHODS

Antecubital vein blood was drawn from apparently healthy 12-hour-fasted subjects of age 25 to 35 years. Blood samples were processed 2 hours after withdrawal.

Lipid Extraction. Serum (15 to 20 ml.) was added dropwise to 25 volumes of 2:1 (v/v) chloroform-methanol. After shaking, the extract was allowed to stand for 30 minutes and then filtered into a 1 l. separatory funnel. Flask and filter paper were washed twice with 20 ml. portions of chloroform-methanol. An equal volume of distilled water was carefully layered on top of the filtrate and allowed to equilibrate for at least 3 hours. Recoveries of lipid phosphorus were unaffected by the addition of salts ($CaCl_2$, $MgCl_2$) to the distilled water. The lower chloroform layer and the fluffy interphase were removed from the upper aqueous methanol layer. The extract was taken to dryness on a flash evaporator under vacuum and kept under nitrogen. The dried residue was taken up in 15 ml. of chloroform or petroleum ether (b.p. 35 to 70°C) and used immediately or stored in a stoppered flask at 4°C for not more than 2 days. All solvents used throughout extraction and chromatography were doubly redistilled from reagent grade material.

Silicic Acid Chromatography. Silicic acid columns were prepared as described by Hirsch and Ahrens (1) using 18 g. activated silicic acid and a water-jacketed assembly allowing maintenance of the column at a constant temperature of 25°C. Uniform packing of the dry column (1.8 × 12 cm.) was accomplished by gentle tapping with a glass rod, followed by firmer packing with an electric vibrator. Dehydrating washings with diethyl ether (10 ml.), 1:1 (v/v) acetone-diethyl ether (30 ml.), and diethyl ether (20 ml.) were followed by conditioning with petroleum ether for 6 to 10 hours before applying the sample.

Columns were re-used after complete stripping of all lipid components with 200 ml. methanol followed by the regular dehydrating washings and petroleum ether conditioning for 10 hours.

Elution was accomplished with 150 ml. diethyl

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[1] Throughout this article the term "cephalin fraction" refers to the corresponding fraction described by Hirsch and Ahrens (1).

ether followed by 500 ml. 1:4 (v/v) chloroform-methanol. Upon addition of chloroform-methanol, fractions of 360 drops were collected by means of an automatic fraction collector. The flow rate from the column was 12 to 20 ml. effluent per minute.

Analyses. Fractions were analyzed for phosphorus by the method of King (5). The samples within each peak were pooled for further analysis. Carboxylic acid ester groups were determined according to Rapport and Alonzo (6). The ferric perchlorate solution used in this method was prepared in n-butanol instead of in ethanol and only 4 ml. of this reagent was used. Amino groups were detected by a ninhydrin method (7). Triglycerides and glycerol were determined according to Van Handel and Zilversmit (8), although complete recovery and measurement of phospholipid glycerol was not possible by this method. Choline was estimated by the method of Glick (9) and cholesterol by the method of Sperry and Webb (10). Hemolytic activity was tested by 3 hours' incubation with 2 ml. of 1 per cent suspension of washed human erythrocytes in isotonic saline at room temperature.

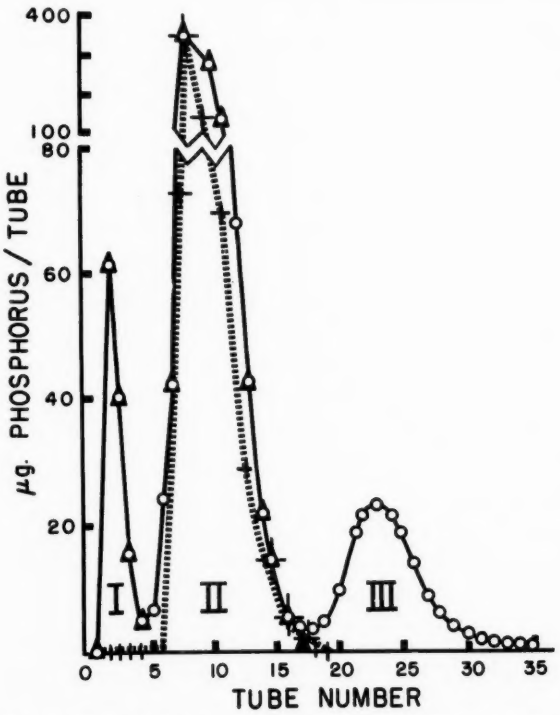

FIG. 1. Elution pattern of phospholipids from silicic acid with 500 ml. 1:4 (v/v) $CHCl_3$—MeOH. Extract from 15 ml. normal human serum: ○; rechromatography of Peaks I and II: ▲; beef spinal cord sphingomyelin (6 mg.) + L-α-dimyristoyl lecithin (6 mg.): + dotted line.

Paper Chromatography. Chromatography was carried out on unimpregnated paper according to Witter *et al.* (11), using 30:7 (v/v) 2,6-dimethyl-4-heptanone-acetic acid. Chromatography on silicic acid impregnated paper was carried out according to Marinetti and Stotz (12), using the n-butyl ether-acetic acid-chloroform-water system and on silicic acid impregnated glass fiber filter paper according to Dieckert *et al.* (13), using the ether-acetone-phenol-water system. Spots were detected with Rhodamine B (11), ninhydrin (11), and iodine vapor.

Infrared Spectra. Infrared spectra were obtained on a Perkin-Elmer Model 13 spectrophotometer using an NaCl prism. Samples were prepared by evaporating chloroform solutions onto an AgCl plate. Samples were run against air as a blank, using the constant energy program.

Materials. Sphingomyelin prepared by the method of Rapport and Lerner (14) was obtained from the Sylvana Chemical Company, Orange, New Jersey. Synthetic L-α-dimyristoyl lecithin and L-α-dimyristoyl phosphatidylethanolamine were obtained from LaMotte Chemical Company, Chestertown, Maryland. Lecithinase A was prepared from commercial pancreatin (Viobin Corporation) by the method of Gronchi (15). Lysolecithin was prepared by the action of lecithinase A on L-α-dimyristoyl lecithin and on beef spinal cord lecithin (Sylvana Chemical Company).

RESULTS

Lipid phosphorus was recovered in the initial extraction procedure to the extent of 88.2 to 97.2 per cent. Lipid phosphorus eluted from silicic acid columns was recovered to the extent of 90 to 101 per cent. Complete recovery of the triglycerides and free and esterified cholesterol was observed in the diethyl ether fraction initially eluted from columns. Only traces of phosphorus were found in this fraction and in the final methanol elution used for stripping the columns.

Figure 1 shows the typical elution pattern of the phospholipids from normal human serum obtained by elution with chloroform-methanol 1:4 (v/v). The three peaks, I, II, and III, were always obtained with the same efficiency of separation. No sign of column overloading was evident, even with the lipid extract from 100 ml. plasma (approximately 175 mg. phospholipid). Poor separation was noted in old columns, probably due to hydration, or when positive pressure was applied during elution, or when column temperature exceeded 25°C.

Table 1 shows the amount of lipid phosphorus in the three peaks expressed as mg. phosphorus per 100

ml. serum and as the percentage of total recovered lipid phosphorus from eight normal serum specimens.

Peak I. As shown in Table 2, the mean carboxylic acid ester bond to phosphorus ratio was 2.01 (1.90 to 2.08). This falls within the range of variation for both determinations when these were performed on standard lipids. Glycerol was present, choline was absent, and a positive ninhydrin reaction was obtained. No hemolytic activity could be demonstrated. This falls within the range of variation for both determinations when these were performed on standard lipids. The Peak I components were subdivided on silicic acid by using 200 ml. chloroform-methanol 7:1 (v/v), followed by 200 ml. chloroform-methanol 4:1 (v/v). These components were revealed by paper chromatography to be principally phosphatidylethanolamine and a trace of phosphatidylserine. Table 1 shows that these cephalins were present to the extent of 0.63 mg. phosphorus per 100 ml. serum and represent 6.4 per cent of the total lipid phosphorus. These values are in good agreement with those given by Phillips (3), Posborg Petersen (16), Sinclair (17), and Hack (18).

Peak II. Table 1 shows that Peak II contained 85 per cent of the total recovered lipid phosphorus and represents 8.3 mg. phosphorus per 100 ml. serum. Table 2 shows that the Peak II material had no hemolytic activity and was ninhydrin-negative when the reaction was carried out in solution. Glycerol was present and the mean ester bond to phosphorus ratio was 1.57 (1.46 to 1.83). The choline to phosphorus ratio was only 0.73. The discrepancy between these ratios suggested a mixture of phospholipids. Figure 1 shows a model experiment in which a mixture of synthetic L-α-dimyristoyl lecithin and beef spinal cord sphingomyelin failed to separate and showed identical elution characteristics to the Peak II material under the conditions used. The infrared spectrum of the Peak II material as compared to synthetic L-α-dimyristoyl lecithin and beef spinal cord sphingomyelin was compatible with a mixture of lecithin and sphingomyelin. Lecithin showed characteristic bands at 1740, 1470, and 1170 cm.$^{-1}$ Sphingomyelin showed principal bands at 1625, 1535, and 1470 cm.$^{-1}$ All five bands were present in the Peak II material.

Paper chromatography revealed the presence of both lecithin and sphingomyelin, and a trace of ninhydrin-reacting material. The method employed for carboxylic acid ester determination in these experiments does not detect the sphingomyelin fatty acid held through the amide linkage since it is apparently not freed by hydroxylamine under alkaline conditions. Under the conditions of 90-minute alkaline hydrolysis described by Glick (9), sphingomyelin choline was not released. The King procedure (5) estimates the phosphorus from both lecithin and sphingomyelin, whereas the Glick procedure (9) estimates the choline and the Rapport and Alonzo method (6) determines the fatty ester bonds from lecithin only. Therefore in a mixture of lecithin and sphingomyelin, both the choline to phosphorus and fatty ester to phosphorus ratios will be lower than the theoretical value for lecithin alone. The carboxylic acid ester to phosphorus ratio of 1.57 therefore allows estimation of these Peak II components on the assumption that the carboxylic acid ester to phosphorus ratio for lecithin is 2.00. This gives in Peak II a mean lecithin content of 78.5 per cent and sphingomyelin content of 21.5 per cent. Corresponding correction of the choline to phosphorus ratio gives a value of 0.93 for lecithin. Thus lecithin represents 66.4 per cent and sphingomyelin 18.4 per cent of the total recovered phosphorus, or 6.54 and 1.79 mg. phosphorus per 100 ml. serum respectively. These values are in good agreement with those reported by previous workers (3, 16, 17, 18).

Peak III. Table 2 shows that this material was ninhydrin-negative and contained glycerol. The mean carboxylic acid ester to phosphorus ratio was 1.11 (1.02 to 1.26) and the choline to phosphorus ratio was 1.17. Hemolytic activity was clearly present with 0.015 μmole of material and detectable with 0.010 μmole. The infrared spectrum differed from that of sphingomyelin as well as from that of the Peak II material but was similar to that of the lecithin standard. Bands were observed at 1740 and 1170 cm.$^{-1}$ but not at 1625 or 1535 cm.$^{-1}$ This indicates that the Peak III material was not contaminated with sphingomyelin. The only differences from the spectrum of lecithin were quantitative rather than qualitative. Nelson and Freeman (19) did not report the presence of lysolecithin in their studies on serum. However, lysolecithin would probably have eluted with the mixed lecithin and sphingomyelin fraction in their solvent systems and the lysolecithin spectrum, which is qualitatively similar to that of lecithin, would have been masked by lecithin. Paper chromatography of the Peak III material yielded a spot having the same mobility as the product of pancreatic lecithinase A action on L-α-dimyristoyl lecithin, beef spinal cord lecithin, or on the Peak II material. The Peak III substance was resistant to the action of pancreatic lecithinase A. It was concluded from the evidence that the Peak III material was lysolecithin, which Table 1 shows to compose 8.9 per cent of the total lipid phosphorus or 0.88 mg. phosphorus per 100 ml. serum.

Figure 1 shows the results of rechromatography of

TABLE 1. PHOSPHORUS CONTENT OF CHROMATOGRAPHICALLY SEPARATED PHOSPHOLIPID COMPONENTS OF NORMAL HUMAN SERUM

Exp. No.	Sex	Mg. lipid P per 100 ml. serum				Percentage of total lipid P		
		I *	II †	III ‡	Total	I *	II †	III ‡
23	M	0.55	7.12	1.03	8.70	6.4	82.1	11.5
27	F	0.63	9.57	0.96	11.16	5.6	85.7	8.6
28	F	0.98	7.35	0.85	9.18	10.7	80.1	9.2
29	F	0.85	10.95	1.07	12.87	6.6	85.1	8.3
40	M	0.64	8.23	1.00	9.87	6.5	83.4	10.1
49	M	0.59	9.44	0.71	10.74	5.5	87.9	6.6
57	F	0.34	6.02	0.60	6.96	4.9	86.5	8.6
62	M	0.42	7.94	0.82	9.18	4.6	86.5	8.9
Mean		0.63	8.33	0.88	9.84	6.4	84.7	8.9
S.D.		±0.21	±1.58	±0.16	±1.78	±1.9	±2.6	±1.4

* Cephalins.
† Lecithin plus sphingomyelin.
‡ Lysolecithin.

TABLE 2. PROPERTIES OF COMPONENT PEAKS OF PHOSPHOLIPIDS FROM SERUM

	Carboxylic acid ester/P	Choline/P	Glycerol	Ninhydrin	Hemolytic Activity	R_f *		
						Solvent A †	Solvent B †	Solvent C †
Peak I	2.01	0	+	+	0	0.47 ± 0.07 0.10 ± 0.10	0.57 ± 0.14 0.66 ± 0.06	0.80 ± 0.17
L-α-dimyristoyl phosphatidyl ethanolamine	−	−	−	−	−	0.55 ± 0.11	0.56 ± 0.12	0.80 ± 0.17
Peak II	1.57	0.73	+	0	0	0.69 ± 0.09 0.40 ± 0.10 0.56 ± 0.04 ‡	0.60 ± 0.09 0.30 ± 0.10 −	0.67 ± 0.13 0.18 ± 0.04 ‡
L-α-dimyristoyl lecithin	−	−	−	−	−	0.70 ± 0.07	0.53 ± 0.11	0.75 ± 0.16
Sphingomyelin	−	−	−	−	−	0.39 ± 0.10	0.36 ± 0.11	0.67 ± 0.18
Peak III	1.11	1.17	+	0	+++	0.41 ± 0.09	0.18 ± 0.10	0.38 ± 0.11
L-α-dimyristoyl lecithin plus lecithinase A	−	−	−	−	−	0.35 ± 0.12	0.16 ± 0.09	0.29 ± 0.14
Peak II plus lecithinase A	−	−	−	−	−	0.25 ± 0.10	0.20 ± 0.10	0.27 ± 0.09

* R_f computed to center of spot ± ½ spot length ÷ distance to solvent front.
† Solvent A = Diisobutylketone/acetic acid (30:7) (11); Solvent B = n-Butyl ether/acetic acid/$CHCl_3$/H_2O (40:35:6:5) (12); Solvent C = Ether/acetone/phenol/H_2O (13).
‡ Ninhydrin positive.

the pooled material in Peaks I and II after taking to dryness and redissolving in chloroform. It may be seen that no Peak III material appeared, which indicates that lysolecithin was not formed during chromatography.

The occurrence and quantity of lysolecithin was unchanged when water washing of the lipid extract was omitted. No significant differences in elution pattern or peak quantities were observed when equal portions of the same dried serum lipid extract were dissolved in chloroform or in petroleum ether. The quantity and elution pattern of the serum phospholipids was unchanged whether serum or the lipid extracts were processed within 2 hours or stored for 1 week at 4°C. No quantitative differences were observed in the peak materials when serum was compared with heparinized plasma from the same subject and using siliconized glassware to prevent breakdown of formed elements.

DISCUSSION

Well-defined separation of three major phospholipid components from human serum was afforded by the use of 1:4 (v/v) chloroform-methanol elution from silicic acid columns. Identification of these peak components revealed them to be different from the three major peaks obtained by Hirsch and Ahrens (1) by methanol elution from a silicic acid column. These workers reported cephalin, lecithin, and sphingomyelin to be eluted, in that order. The present study indicates that the peaks are cephalin, lecithin and sphingomyelin, and lysolecithin, in that order. The finding of lysolecithin in human serum offers confirmation to similar reports by Phillips (2, 3). In earlier experiments some separations of lecithin and sphingomyelin were accomplished with chloroform-methanol 3:2 (v/v), although this separation was more effective when followed with chloroform-methanol 1:1 (v/v). No other combination of chloroform and methanol, nor methanol alone, effected separation between lecithin and sphingomyelin.

Paper chromatography revealed the presence of minor amounts of a ninhydrin-reacting substance in the Peak II material, which appeared only when the spot was concentrated on filter paper but which did not react with ninhydrin in solution. This material did not give positive reactions with Rhodamine B or iodine vapor. This may be similar to the unidentified ninhydrin reacting substance noted by Phillips (3) and may represent as yet undescribed lipid.

The origin of lysolecithin in serum is not clear at present. Control experiments render it unlikely that it is formed during extraction, purification, or chromatography of the extract. It may be possible that it is formed shortly after the blood is drawn, although there is no evidence to favor this possibility.

It is of interest that Gray and Macfarlane (20) reported the production of lysophosphatides from the corresponding plasmalogens when chloroform-methanol extracts of ox heart were chromatographed on silicic acid without prior removal of the neutral fat fraction. In the studies reported here, acetone extraction of neutral lipids, and silicic acid chromatography of the acetone-insoluble phospholipids showed the same three peaks in the same relative amounts.

Hajdu *et al.* (21) reported the chemical isolation and purification of β-palmitoyl lysolecithin from plasma and also from liver, heart, and adrenal medulla. These workers found that 50 per cent of the lysolecithin occurred with the α-position bound in a hemiacetal linkage with a fatty aldehyde. This was converted to β-lysolecithin on allowing the extract to stand at pH 2.0 for 24 to 48 hours. It was also noted that lysolecithin was decomposed on silicic acid only in the presence of aqueous solvents.

Studies [2] on a number of patients suffering from various disorders showed a marked elevation both in serum lysolecithin and serum amylase in acute pancreatitis without a simultaneous increase in total phospholipid concentration. This suggests that lysolecithin is indeed a normal constituent of serum.

It is not certain whether plasma lysolecithin may be a precursor or a breakdown product of lecithin or neither. Borgström (22) has reported the presence of lysolecithin in intestinal contents but it is not known whether this is absorbed as such.

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Isolation and determination methods for brain cerebrosides, hydroxy fatty acids, and unsaturated and saturated fatty acids *

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SUMMARY

A new method is described for the isolation of nearly pure total cerebrosides from fresh brain. Florisil columns are used to separate the cerebroside hydroxy and normal acids, in the form of their esters, and to separate the saturated and unsaturated esters of each group. The esters within each class are determined by gas chromatography, the hydroxy esters being run as the methyl ethers. Yields in the various steps are close to theoretical. The presence of lower homologues in all four classes of rat brain cerebroside acids was discovered. Particularly large amounts of C_{23} and C_{22} saturated acids were found.

Although the major fatty acids occurring in brain cerebrosides were identified over 30 years ago, mainly through the efforts of Klenk and Thierfelder, as reported by Deuel (1), the percentage of each acid has never been determined except in a rough way by fractional isolation of the different cerebrosides (2). To make such an analysis it is necessary to isolate the cerebrosides quantitatively, so that no enrichment of one type occurs during the isolation, and in pure form, free from other compounds containing fatty acids. The analysis of the fatty acids from cerebrosides is a problem in itself, complicated by the presence of very lengthy chains and hydroxy groups.

This paper describes the isolation of cerebrosides by an extension of our previously described method (3), in which the lipids of whole brain are applied to a Florisil [1] column, the cholesterol is eluted with ether, and the cerebrosides are eluted with chloroform-methanol. A contaminating ester (4) is saponified with alkali, and the reaction products are removed with water and ion exchange resins. The cerebroside sulfate is removed in the latter step.

The cerebroside acids are liberated by methanolysis and the esters are separated by chromatography into two groups: normal and hydroxy esters. The two groups are each divided into saturated and unsaturated esters by formation of mercurial addition products and Florisil chromatography. The four groups of esters are then analyzed by means of gas chromatography.

It is believed that the methods described here are of use in other analytical and isolation problems involving cerebrosides and fatty acids.

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[1] Floridin Co., Tallahassee, Fla.

EXPERIMENTAL

Materials. A simple glass still was used to redistill nearly all the solvents: ACS absolute methanol, USP chloroform, ACS toluene, 95 per cent ethanol, and petroleum ether b.p. 66-7°C (Skellysolve B). Absolute ether, used for chromatography, was freshly redistilled from KOH pellets; USP ether was used for extractions.

Florisil, purchased as 60 to 100 mesh, was resieved to 70 to 100 mesh and stored in glass bottles. If the sieving is done in hot humid weather, adsorptive power is partially lost and reactivation is necessary. This is done by heating the adsorbent 1 hour at 650°C. Dowex 1-X2 anion exchange resin (200 to 400 mesh) was cycled and converted to the hydroxyl form; Dowex 50-X4 (200 to 400 mesh) was cycled

and converted to the hydrogen form. The resins were mixed in the proportion 7:2, based on moist weight.

Lignoceric acid was prepared by the method described by Carroll (5), and from this, DL-cerebronic acid (m.p. 97°-98°C), by bromination and hydrolysis (6). The previously reported melting point for DL-cerebronic acid was 99.5°-100°C (7). Our preparation of methyl DL-cerebronate melted at 74.5°-76°C, in contrast to the reported melting point of 65°C for the D-cerebronate (8). Samples of lignoceric acid, nervonic acid, and methyl α-hydroxybehenate were generously supplied by Dr. K. K. Carroll. Pure methyl oleate, palmitate, and stearate were obtained from the Hormel Foundation.[2] Commercial grades of α-hydroxystearic, arachidic, and behenic acids and also methyl myristate and laurate were used as identification standards for gas chromatography.

Equipment. The column techniques were described previously (9). Evaporations were performed under vacuum with a swirler evaporator (10) or rotary evaporator. Gas chromatography was performed with an Aerograph fitted with a thermal conductivity detector and a 30-inch copper column (¼ inch O.D.).[3] The packing for the column contained the polytetramethylene glycol-succinate ester described by Dr. B. M. Craig, to whom we are indebted for information about this material. The samples of esters for analysis were dissolved in about 3 volumes of xylene and about 10 microliters of solution were injected into the column with a micro-syringe.[4]

Isolation of Crude Cerebrosides. A group of from 11 to 30 rat brains was homogenized with 20 volumes of chloroform-methanol (2:1) according to the method of Folch *et al.* (11); the mixture was filtered, and the extract was taken to dryness in a rotary evaporator. Toluene was added near the end of the evaporation to prevent splashing. The lipids were dried overnight in an evacuated desiccator over KOH and wax. To ensure cleavage of the proteolipids, the extract was refluxed 45 minutes with six volumes of chloroform-methanol and filtered, evaporated to dryness, and again dried in a desiccator.

From 45 to 120 grams of Florisil (30 g. per g. of lipid) were swirled briefly under vacuum with absolute ether, and the slurry was poured into a column 2.2 to 2.8 cm. I.D., yielding a packing about 45 cm. high. The crude lipids were warmed with ether (40 ml. per g. of lipids), and the suspension was swirled under vacuum with more Florisil (10 g. per g. of lipids). This mixture was added to the column, together with two rinses of ether, and elution was continued with the same solvent (1,000 ml. per g. of lipids). This fraction includes the lipids of low polarity, particularly cholesterol.

The crude cerebrosides were next eluted with chloroform-methanol 4:1 (1,000 ml. per g. of lipids). Evaporation to dryness yielded a white powder which took on a slight yellow tint on storage in a desiccator.

The flow rate was 400 to 500 ml. per hour for an 80 g. column (2.2 cm.). In early experiments we found that the column tended to form bubbles, but this was prevented by applying slight air pressure on top and a slight constriction below (with Neoprene tubing and a screw clamp). Addition of the lipids suspended in ether, without Florisil, resulted in clogging and bubble formation. Stopping the column flow for any extended period also tends to produce bubbles.

Removal of Ester and Ionic Lipid. The crude cerebrosides were dissolved in 450 volumes of methanol (based on cerebroside weight), and 45 volumes of aqueous NaOH (40 g. per 45 ml. water) were added with cooling. After stirring and heating at 37°-39°C for 3 hours, phenol red was added, and then concentrated HCl (with cooling) until just past the orange end point. The mixture was evaporated to dryness and dried over P_2O_5. The residue was extracted with warm chloroform-methanol (2:1) while crushing the salt cake with a glass rod, the salt was filtered off, and the filtrate was washed with water in a centrifuge tube to extract the alcoholic portion of the ester. Probably some cerebroside sulfate was lost in the water (12).

The chloroform and interface material were evaporated to dryness, the lipids were dissolved in ethanol-toluene-water (7.5:6.1), and the ionic lipids were removed by passing the solution through a column of mixed ion exchange resins. The column packing was 1×35 cm.; the resins were prewashed in the column with 250 ml. of the same solvent; the sample was added in the course of 1 hour, and elution was carried out with 250 ml. of the same solvent in the course of 1 to 2 hours. The effluent was evaporated to dryness and dried further in a desiccator. Samples of these cerebrosides were drawn for galactose determination by the phosphoric acid-anthrone method (3).

Methanolysis and Separation of Normal and Hydroxy Acids. The cerebrosides (130 to 300 mg.) were dissolved in 20 ml. of Mg-dried methanol saturated with HCl at 0°C, and refluxed 16 hours with an oil bath set at about 95°C. The resultant esters were extracted with four 15 ml. portions of Skellysolve B, 30 ml. of ether were added to the pooled extracts, and the solution was washed with water five times. The yield of

[2] Austin, Minn.
[3] Wilkens Instrument and Research, Inc., Walnut Creek, Calif.
[4] Hamilton Company, Whittier, Calif.

dry esters ran a few per cent too high, probably because of the extraction of some sphingosine derivatives.

Eight grams of Florisil in Skellysolve B were packed into a 1.1 cm. I.D. column, and the mixed esters were added in 20 ml. of the same solvent. Elution with 1,600 ml. of the petroleum ether yielded the normal (unhydroxylated) esters. The hydroxy esters were eluted with 1,200 ml. of Skellysolve B-absolute ether (9:1). The flow rate was about 400 ml. per hour. The two effluents were evaporated to dryness, dried further under vacuum, and weighed.

Separation of Saturated and Unsaturated Esters. The dry esters were heated in a screw-cap test tube (Teflon-lined cap) for 2 hours at 66°C with about four mole equivalents of mercuric acetate and 15 volumes of methanol (based on the weight of the acetate). The mercuric acetate was first dried over P_2O_5. Under these conditions the unsaturated esters form the following polar grouping: —CH(HgOAc)—CH-(OCH_3)— by addition to the double bond (13). The reaction mixture was transferred to a separatory funnel with 50 ml. of ether and washed with two portions of acetic acid-water (1:30). Five additional washes with water completed the removal of the excess reagents. The ether layer was evaporated to dryness and stored in the dark over P_2O_5. The other steps were carried out under reduced light conditions.

In the case of the normal esters, the sample was dissolved in 5 ml. of Skellysolve and passed through a 0.6 cm. I.D. column containing 2 g. of Florisil in the same solvent. Elution with 410 ml. of Skellysolve yielded the saturated esters, and the unsaturated esters were eluted with 50 ml. of ethanol-chloroform-concentrated HCl (10:8:1). The HCl re-forms the double bond in the original configuration (14), and the polar solvent readily elutes the ester. To minimize the possibility of hydrolysis of the ester by exposure to acid, we caught the effluent in a flask containing 70 ml. of water and 50 ml. of ether. The organic layer was washed well with water to remove mercuric salts and also a good deal of inorganic material arising from the Florisil ($MgCl_2$?). The esters were dried and weighed. In the case of the hydroxy esters, the saturated esters were eluted with Skellysolve-ether (9:1).

Gas Chromatography of the Esters. The above separations into four groups of esters were carried out because the column that was available could not separate the individual esters sufficiently well. It was found that the hydroxy esters did not come off the column at the maximum temperature attempted (217°C), so the methyl ethers were prepared and found to give satisfactory curves. The C_{22} methoxy ester came off the column at the same time as the C_{24} normal ester.

Methylation was performed by refluxing about 35 mg. of hydroxy ester with 1 ml. of redistilled methyl iodide and 200 mg. of dry silver oxide. The mixture was stirred with a magnetic stirrer and heated with a water bath. After 30 minutes another portion of iodide and oxide was added, and then, after 60 minutes more, another portion of oxide. Heating and stirring were continued for a total period of 3 hours, toluene was added, the mixture was filtered, and the solids were washed well with toluene. The filtrate was evaporated to dryness and dissolved in xylene for injection into the column. The ethers were chromatographed at about 216°C and the normal esters at about 206°C. The helium flow rate was about 105 ml. per minute for the ethers and 90 ml. per minute for the normal esters.

RESULTS AND DISCUSSION

Isolation of Purified Cerebrosides. When the cerebroside elution from Florisil is followed by examination of successive portions of effluent, it is seen that the content of white solid (crude cerebrosides) decreases by long trailing to a very low level. At this point a small amount of oily material starts to come off, with perceptible overlapping. In the procedure used for this study, some of this oil is included in the crude cerebrosides. The elution of the oil is greatly speeded by use of chloroform-methanol (2:1). The yield of oil was 12.8 mg. per brain in 81 g. rats, and 17.5 mg. per brain in 449 g. rats, so it can be seen that this fraction is one of the more plentiful brain lipids. The oil and the ether effluent from the oldest group of rats were hydrolyzed with 2 N HCl and the galactose contents determined by an anthrone procedure (15). No galactose was found in the ether effluent, but an amount corresponding to 3.4 per cent of the cerebroside galactose was found in the oil. It is likely that this glycolipid is not trailing cerebroside, but rather Fraction III isolated by Weiss (16). Fraction III, for which we suggest the name neuroside, is a glycolipid which strongly resembles a cerebroside but is more polar, and contains an amino compound which becomes water-soluble on acid hydrolysis.[5]

The use of saponification for further purification of the crude cerebrosides was prompted in part by our observation that a degradation product formed during purification of cerebrosides by reaction with $Ba(OH)_2$ (15), and in part by the observation of an ester ab-

[5] Unpublished work, N. S. Radin and M. V. Kelley, Jr.

sorption peak by Burton *et al.* (4). The saponification step was investigated with conditions of increasing severity, using a sample of crude cerebrosides from spinal cord (15) and evaluating the results from the yields of cerebrosides and their galactose contents. The very mild conditions of Dawson (17) were inadequate, and we turned to the use of almost 2 N NaOH. Heating 1, 3, and 6 hours resulted in yields of 77.6, 76.7, and 75.0 per cent, while the galactose content of all three samples was very similar. Apparently there is a very slow breakdown of cerebrosides (psychosine would stick to the ion exchange resins), so a 3-hour saponification was accepted as a reasonable time. The cerebroside sulfate that is present in the crude cerebrosides does not lose the sulfate group during saponification; such a reaction would show up as an increasing rather than decreasing yield of cerebroside. We had previously shown the efficient uptake of lipid sulfates by ion exchange resins with the aid of sulfur-labeled lipids (12). The galactose contents of the crude and purified cerebrosides were compared in the cases of two groups of rats, and the differences in contents were calculated as cerebroside sulfate (Mg salt). The sulfatide contents per brain came out 2.25 and 9.0 mg. for groups B and D (see accompanying paper). The sulfatide to cerebroside ratios are 0.25 and 0.41, in reasonable agreement with the estimate made by Blix (18).

The effectiveness of the ion exchange resins for removing the fatty acids resulting from saponification was tested by addition of C^{14}-palmitic acid to the saponification mixture. Using a column of resins 0.6 $\times$ 15 cm., it was found that 4.2 per cent of the radioactivity leaked through, so the amount of resins was raised almost sevenfold. Later work with this palmitic acid showed that the leaking material was a nonionic radioactive impurity.

The galactose contents of the resultant cerebrosides in a series of five isolations ranged from 20.6 to 21.4 per cent. The theoretical galactose content, assuming (19, 20, 21) the cerebrosides are monohydrates of phrenosine (21.3 per cent galactose) and kerasine (21.7 per cent galactose) in the ratio 60:40, is 21.46 per cent. Allowing for the presence of shorter fatty acids might bring this up to 21.6 per cent. On this basis our preparations were 95 to 99 per cent pure. The nature of the impurity is unknown.

The actual yield of cerebrosides was not rigorously determined, but it appears to be practically quantitative. Previous work with brain cerebrosides (3) had shown that there is complete recovery when the Florisil is eluted with chloroform-methanol (2:1). Complete recovery is also obtained when a sample of cerebrosides is reprocessed through the saponification and ion exchange steps.

The Methanolysis Step. As indicated by Rosenheim (20), methanol-sulfuric acid was found to yield free acid as well as methyl esters. Allowing the refluxing vapors to percolate through silica gel to remove water prevented formation of free acids but the yield of esters was low. Possibly the esterification of methanol with sulfuric acid is a strongly competing reaction. After studying various conditions, it was decided that a lengthy treatment with maximal HCl concentration in methanol was best. This gives too high a yield of petroleum ether-extractable lipid, but the excess material is strongly adsorbed by Florisil and is separated from the esters when the normal and hydroxy esters are separated. Thus the yields of crude esters from five rat groups were 54.0, 52.5, 52.9, 50.2, and 52.6 per cent of the cerebroside weights. After chromatography, the normal plus hydroxy esters amounted to 45.7, 45.7, 46.3, 48.9, and 47.6 per cent of the cerebroside weights. The expected value is about 47 per cent.

Separation of Normal and Hydroxy Esters. Early attempts at separating the normal and hydroxy esters by converting the latter to hemisuccinates (22) or sulfates (23) and separating the derivatives by solvent partition led to incomplete separation. Florisil was found to be a suitable adsorbent and was tested with separate esters (stearate, lignocerate, cerebronate, and hydroxystearate) and with known mixtures. The maximal loading of hydroxy esters is roughly 10 to 15 mg. per g. of adsorbent; with higher loads some leakage into the normal ester fraction is observed. The recoveries are excellent provided the long trailing typical of Florisil is overcome by adequate volumes of eluting solvent. The trailing should be reduced by use of finer mesh powder. A small amount of material comes off the adsorbent, but this is largely left behind on the glassware when the effluent is evaporated to dryness and the esters are redissolved in petroleum ether and transferred. The blank thus obtained usually is about 0.05 mg. per g. of adsorbent.

Of general interest is the finding that cholesterol is eluted with the hydroxy esters. This means that Florisil can be used to replace alkaline saponification for the separation of cholesterol from fatty acids, provided there is no appreciable amount of hydroxy fatty acid present. In other words, one can split a lipid sample by methanolysis, extract the esters and cholesterol with petroleum ether, and separate the fatty esters and cholesterol with a Florisil column. Where very long fatty acids are present (such as lignoceric), or excessive emulsification and pigment formation occur, this method is superior to solvent separation of "sa-

TABLE 1. GAS CHROMATOGRAPHIC ANALYSIS OF KNOWN ESTER MIXTURES

	Palmitate	Arachidate	Lignocerate
	percentage	*percentage*	*percentage*
Mixture 1			
Known composition	23.0	22.8	54.2
Observed compositions	24.4	23.8	52.0
	23.6	24.8	51.6
	24.6	22.9	52.5
Mixture 2			
Known composition	18.6	40.8	40.6
Observed compositions	18.2	41.0	40.8
	18.6	40.5	40.9
Mixture 3			
Known composition	23.5	55.4	21.1
Observed compositions	23.4	54.5	22.2
	22.9	56.0	21.1

ponifiables" and "unsaponifiables." Free fatty acids and most pigments are adsorbed very strongly by Florisil and can be separated from cholesterol.

Separation of Saturated and Unsaturated Esters. The use of the mercury addition products for separation of these esters by paper chromatography was described by Inoue *et al.* (24), and very recently a note has appeared describing the use of a silica gel column (14). Our method was checked [6] with 48.3 mg. of pure methyl oleate and a 3-g. Florisil column. The yield of material coming off with petroleum ether was 0.9 mg. (corrected for a blank of 0.56 mg.), and the yield of material eluted by the acidic solvent was 47.7 mg. (99 per cent). Assay by a modified hydroxamic acid method (25) indicated the product was 97.4 per cent ester.

Another test, with 25.2 mg. of methyl cerebronate resulted in recovery in the hydroxy ester fraction of 24.8 mg. of material having the original melting point. Apparently our procedure results in losses of about 2 per cent.

Preparation of the Methyl Ethers. A test run with 111.2 mg. of methyl DL-cerebronate resulted in a yield of 115.3 mg. of ether (100 per cent). Of course a weight measurement gives an unprecise indication of completeness of methylation. A methoxyl determination on this material [7] gave a value of 14.61 per cent methoxyl (theoretical is 15.01 per cent). Two recrystallizations of the ether from methanol yielded ether assaying 14.80 per cent methoxyl; this melted at 54°-55°C the first time and subsequent melting points were 48.7°-49°C. The melting point of the natural (D) isomer was reported as 58°-59°C, but methoxyl values ranged from 14.06 to 14.72 per cent (26).

Our first attempts at forming the ether were with distilled diazomethane and the ester or free acid, but the yields obtained on evaporation to dryness were somewhat above theoretical.

Gas Chromatographic Analysis. To identify the elution peaks, standard known mixtures were run before each analysis period. Retention times, measured from the air peak, varied slightly from run to run. The retention times were plotted against the number of carbon atoms on semilog paper for each run, and the line obtained from the standard run was shifted slightly for maximal overlapping. It was found that the composition of the mixture affected the retention time: a component occurring in relatively high concentration came off more slowly, while a minor component tended to come off a little early.

The semilog plot for the unsaturated normal esters was parallel to that of the saturated esters. Since no standards were available for the unsaturated hydroxy esters, we simply used the plot from the saturated methoxy esters and shifted it to coincide with the major (C_{24}) peak.

The percentage of each major component was calculated from the ratio of its area on the elution curve to the total area for all the eluted peaks. The area of each well-separated peak was assumed to be the peak height times the width at half the peak height (27). The area of the poorly separated peaks was determined by weighing a trace of the entire section. The separation and quantitation of the shorter acids could have been improved by use of a lower temperature and longer column, but these (except in the case of the saturated normal acids) constitute a very small proportion of the acids found.

The precision of the quantitative analyses was estimated by analyzing mixtures of known composition made from palmitate, arachidate, and lignocerate. The lignocerate contained 1.9 per cent behenate; the arachidate, 1.8 per cent stearate. These values were used to correct the weights in the standard mixtures. Analytical results are shown in Table 1. A 2 per cent error is common, but some errors reach 5 per cent. The reported values for the unknowns are derived from three runs, so the largest deviation is probably no more than 2 per cent. Similar tests with standard mixtures of saturated methoxy esters showed similar variability.

Typical curves obtained with the four groups of esters are shown in Figures 1 to 4. The very early peaks from the solvent were omitted from the drawings. The numbers above the peaks identify the num-

[6] By A. Hajra, in this laboratory.

[7] By Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

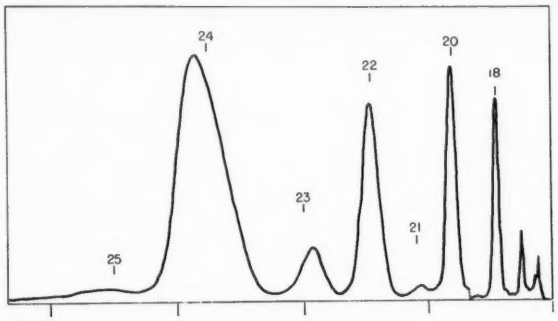

FIG. 1. Gas chromatogram recordings of saturated normal methyl esters obtained from rat brain cerebrosides. The short vertical lines on the abscissa represent 10-minute intervals. The dotted line just after stearate indicates a fourfold increase of sensitivity in the recorder.

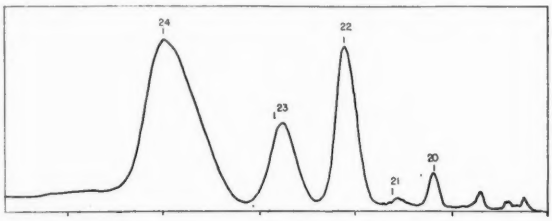

FIG. 2. Gas chromatogram recordings of saturated methoxy methyl esters from rat brain cerebrosides.

ber of carbon atoms in the acid, and the short vertical line just below each number gives the expected peak position.

There is a marked similarity between the two saturated groups and also between the two unsaturated groups. The unsaturated acids contain primarily the known C_{24} compounds, nervonic and α-hydroxynervonic acids, whereas rather large amounts of the C_{23} and C_{22} acids appear in the saturated groups. All four groups contain distinct amounts of the lower homologues, down to C_{16} or less. The shorter acids seem to include isomers, possibly with branched methyl groups, or, in the case of the unsaturated acids, with several double bonds. The similarities in distribution between the hydroxy and normal acids of both types strongly suggest a precursor-product relationship. Further studies on this problem with C^{14}-labeled lignoceric and cerebronic acids are under way.

In the case of the normal acids, it seems likely that part of the lower homologues arises from the impurity known to be present in small amounts in our cerebroside preparations. This explanation is supported by the finding that different preparations yielded somewhat varying contents of the shorter acids. However, Klenk has reported the occurrence of small amounts of unsaturated shorter acids in brain cerebrosides (28, 29). The presence of a longer unsaturated acid, possibly C_{26}, was also reported. Our gas chromatographic column would not separate the Δ^{17} positional isomer of hydroxynervonic acid that was found by Klenk and Faillard (30). We found that there were one or two peaks for acids longer than C_{24} in all four fractions, although the particular curve for unsaturated normal acids chosen for redrawing does not show this. The positions of the peaks do not correspond to straight-chain C_{25} or C_{26} acids, but it is possible that serious shifting occurs with traces of such long acids. In any event, the amounts of longer homologues are very low. The occurrence of behenic acid in a cerebroside from the spleen of a patient with Gaucher's disease has also been reported (31).

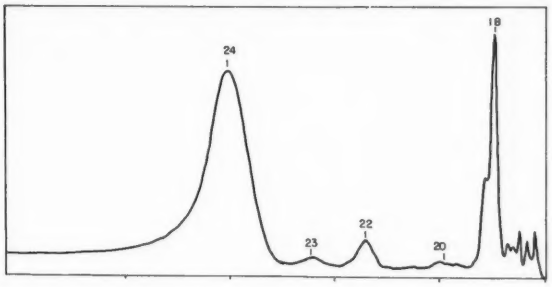

FIG. 3. Gas chromatogram recordings of unsaturated normal methyl esters from rat brain cerebrosides.

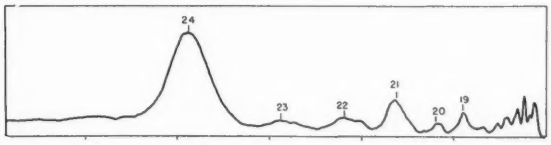

FIG. 4. Gas chromatogram recordings of unsaturated methoxy methyl esters from rat brain cerebrosides.

The existence of C_{22} and C_{26} hydroxy acids, as well as C_{24}, in cerebrosides has been claimed on the basis of X-ray diffraction analyses (32, 33). Wool fat has been shown to contain α-hydroxy acids 10 to 18 carbon atoms long (34) and very recently Skipski *et al.* (35) have found hydroxystearic acid in beef spinal cord cerebrosides. Some of our less pure lipid preparations yielded much higher contents of hydroxypalmitic and hydroxystearic acids, and the origin of these is under study here.

The actual content of each cerebroside acid varies considerably with age; data describing this variation are presented in the article following (36), page 79.

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Volume 1
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Composition of cerebroside acids as a function of age*

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SUMMARY

The brains of from 11 to 30 rats of various age groups—23 to 418 days old—were pooled and analyzed for total lipids, total cerebrosides, and the individual cerebroside acids. Cerebroside deposition is evident over the range of ages studied, and its contribution to the total deposition becomes increasingly important with increasing age. Cerebronic acid is by far the major cerebroside acid, but appreciable amounts of the α-hydroxy C_{23} and C_{22} acids are also present. The unsaturated acids constitute a minor element, and the contribution of the hydroxy unsaturated acids is least. The odd-numbered acids show the greatest increases with age, compared to the other acids, while the hydroxy unsaturated acids show little accumulation except during the earliest period studied. Degradation experiments with the saturated hydroxy acids show that the hydroxyl groups are in the alpha position.

The brains of rats of five different age groups have been analyzed for their contents of total lipids, total cerebrosides, and the individual major cerebroside acids. In the period of life covered—approximately the first half of the life span—marked differences in the contents and deposition rates of the individual cerebroside acids were found.

EXPERIMENTAL

The analyses were carried out as described in the accompanying paper (1). Male rats of the Sprague-Dawley strain [1] were obtained in groups of from 11 to 30 rats. Since the exact ages of each group were unknown, we report only the average body weights. The approximate ages of the groups were 22, 31, 65, 170, and 418 days. The animals were killed with ether, and the entire brains were removed and kept frozen until they could be pooled, weighed, and extracted.

RESULTS AND DISCUSSION

Table 1 shows the values obtained with the lipids and cerebrosides. Each time-point reveals that an increase in brain and cerebroside weight has occurred in the interval. Cerebroside becomes an increasingly important fraction of the brain, even in the middle age period, when the brain weight has almost stabilized and the lipid content has actually started to decrease. The findings of Folch and co-workers (2) with mouse cerebrosides are similar to our results, but there the brain weight appears to fall off at a rather early age (after 90 days). Bürger's data on human brain indicate that cerebroside deposition stops at about 16 years and brain weight starts to fall at about 30 years (3). It would appear that the rodent brain matures more slowly than the human brain with respect to reaching the cerebroside plateau. The methods used by Folch and Bürger for determining cerebrosides were not as specific as ours, and the increasingly large contribution of cerebroside sulfate to the analytical values would probably exaggerate the increases they observed.

Table 2 shows the results of the fractionation of the cerebroside acids into four groups. The percentage distribution data show that the saturated hydroxy acids constitute by far the major group. Somewhat lower are the saturated normal (nonhydroxy) acids, then the unsaturated normal acids, and the unsaturated hydroxy acids constitute the smallest group. Our data confirm the estimates of Klenk (4).

The effect of age on the saturated acids is to decrease the importance of the normal acids during the

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[1] Holtzman Rat Co., Madison, Wis.

second month of life. The saturated hydroxy acids, after showing a slight dip in the second month, rise to even higher values. The normal unsaturated acids are quite constant in percentage, but there is a small drop in later life. The unsaturated hydroxy acids show a rise during the fourth week of life but decline in percentage during the remaining period covered. Thus it is apparent that each of the four groups of acids is affected differently by age.

Looking at the actual weights of the four groups, we see that there is a considerable increase in weight in all the categories. This increase continues throughout the period covered except for the unsaturated acids, which start to decrease in the last stage. The rate of accumulation of the unsaturated hydroxy acids seems to slow down first, and relatively little is deposited after the surge which comes during the fourth week of life.

Contents of the Individual Cerebroside Acids. Tables 3 and 4 list the amount of each of the longer saturated acids. Next to each weight is a number, obtained by dividing the weight at that point by the weight in group A. Thus the number indicates the relative increase in each particular acid with age.

All the acids, except lignoceric, show a continuous rise with age. Lignoceric deposition seems to cease during the fourth week and the third through fifth months, but speeds up considerably in later life. The shorter normal acids, mainly stearic and palmitic, vary erratically, and part of these probably arise from the impurity known to be present in the isolated cerebrosides. The shorter hydroxy acids, on the other hand, increase regularly with age and are probably all authentic members of the cerebroside acids. They comprise only 3 to 4 per cent of the hydroxy acids. The percentage increase is least for lignoceric and behenic acids, and greatest for the odd-numbered acids within each group. Within the hydroxy group, the C_{21} acid shows the greatest percentage increase and the C_{23} acid reaches a level higher than that of lignoceric (at point D). At this age the three most plentiful acids are the $C_{22, 23, 24}$ hydroxy saturated acids.

Table 5 shows the data for the unsaturated acids of two groups of rats. Here, by far, the major components are the C_{24} acids. The normal acids include an appreciable quantity of oleic acid, part of which may be from an impurity. The acid showing the greatest increase with age is the C_{21} hydroxy acid, while hydroxy nervonic acid accumulates only slightly during the second to sixth month. The marked percentage increase of the odd-numbered saturated acids in later life is not too noticeable here.

Saturated and unsaturated acids differ markedly in that the latter are almost entirely C_{24} acids, while the former contain more shorter acids. Comparing within the two groups the distribution of saturated acids, we see that the third most plentiful acid is the C_{23} hydroxy acid and the C_{20} normal acid. In general, it appears that the content and position in the dis-

TABLE 1. CEREBROSIDE CONTENT OF BRAINS AS A FUNCTION OF RAT WEIGHT

	Rat Group				
	A (26 rats)	B (30 rats)	C (11 rats)	D (12 rats)	E (15 rats)
Rat weight, g.	51.2	80.6	243	449	476
Brain weight (wet), g.	1.48	1.61	1.92	2.09	2.18
Total lipids, mg. per brain	106	134	177	210	194
Purified cerebrosides, mg. per brain	5.05	8.9	14.6	21.8	24.0
Cerebrosides as percentage of total lipids	4.8	6.7	8.3	10.4	12.4
Galactose content of cerebrosides, percentage	20.8	21.0	21.0	21.4	20.6

TABLE 2. DISTRIBUTION OF THE CEREBROSIDE ACIDS *

	Rat Group				
	A	B	C	D	E
Percentage Composition of the Total Cerebroside Acids					
Saturated normal acids	30	28	20	20	24
Unsaturated normal acids	11	13	12	12	10
Saturated hydroxy acids	52	47	59	61	62
Unsaturated hydroxy acids	6.8	12.1	9.1	6.7	4.8
Amounts per Brain, in mg.					
Saturated normal acids	0.68	1.09	1.30	1.97	2.60
Unsaturated normal acids	.25	.51	.75	1.17	1.05
Saturated hydroxy acids	1.17	1.82	3.74	5.90	6.88
Unsaturated hydroxy acids	.15	.48	.58	.65	.53
Total	2.25	3.89	6.36	9.68	11.05

* For further description of the five rat groups see Table 1 and text.

TABLE 3. DISTRIBUTION OF THE SATURATED NORMAL ACIDS *

Number of C Atoms	Rat Group				
	A	B	C	D	E
24	285 *1.0*	287 *1.0*	580 *2.0*	585 *2.1*	1099 *3.9*
23	19 *1.0*	26 *1.4*	60 *3.2*	107 *5.6*	127 *6.7*
22	109 *1.0*	131 *1.2*	174 *1.6*	217 *2.0*	264 *2.4*
21	5 *1*	4 *1*	11 *2*	14 *3*	26 *5*
20	37 *1.0*	61 *1.6*	113 *3.0*	209 *5.6*	246 *6.6*
under 20	229	576	357	835	836

* Values for the acids refer to the micrograms of acid per rat brain. Numbers in italics are ratios indicating the increase in content of each acid.

TABLE 4. DISTRIBUTION OF THE SATURATED HYDROXY ACIDS *

Number of C Atoms	Rat Group				
	A	B	C	D	E
24	715 *1.0*	1080 *1.5*	2208 *3.1*	3420 *4.8*	4080 *5.7*
23	66 *1.0*	119 *1.8*	390 *6.0*	875 *13.5*	972 *14.8*
22	342 *1.0*	536 *1.6*	925 *2.7*	1309 *3.8*	1352 *4.0*
21	4 *1*	4 *1*	23 *6*	36 *9*	94 *24*
20	18 *1.0*	32 *1.7*	93 *5.1*	157 *8.7*	179 *9.9*
under 20	29	50	100	105	201

* Values for the acids refer to the micrograms of acid per rat brain. Numbers in italics are ratios indicating the increase in content of each acid.

TABLE 5. DISTRIBUTION OF THE UNSATURATED CEREBROSIDE ACIDS *

Number of C Atoms	Rat Group			
	Normal Acids		Hydroxy Acids	
	B	D	B	D
24	346	866	374	422
23	8	22	15	36
22	22	43	37	48
21	3	5	10	64
20	9	33	4	13
under 20	121	196	37	64

* Values for the acids refer to the micrograms of acid per rat brain.

tribution pattern of each cerebroside acid undergo highly characteristic changes with age. One possible explanation for this individuality is that the distribution varies greatly with location in the brain and the developmental changes in each section of the brain are highly characteristic. Alternatively, the difference may be on a cellular level rather than brain area. It is planned to analyze the cerebrosides of different areas when a less laborious method can be devised.

Identification of the Saturated Hydroxy Acids. These acids were characterized further to demonstrate that they are indeed α-hydroxy acids. A sample of the methyl esters (37.4 mg.) was saponified with KOH-alcohol-benzene (5) for 4 hours at 83°C in a screw-cap test tube (6), and the free acids were obtained by acidification and ether extraction. The dried acids were heated for 2 hours at 42°-43°C with 3 ml. of oxidation solution in a flattened test tube protected with a $CaCl_2$ tube. The oxidation solution consisted of 1 g. of dried potassium acetate, 1 g. of lead tetraacetate (recrystallized from acetic acid), and 20 ml. of acetic acid (redistilled after refluxing about 5 hours with $KMnO_4$). The potassium salt speeds the oxidation (7). The mixture was stirred magnetically until the hydroxy acids dissolved. Test runs with synthetic cerebronic acid gave the theoretical yield of CO_2 under these conditions. The reaction was stopped by adding 7 ml. of a solution made from 1 g. of KI, 5 g. of sodium acetate, and 10 ml. of water, which reduced the tetraacetate and formed iodine (8). About 50 ml. of water was added to dissolve the lead iodide. The iodine was reduced with 10 per cent aqueous thiosulfate, the aldehydes were extracted with ether, and the ether was washed with water and evaporated off. The aldehydes were promptly dissolved in 4 ml. of acetic acid, and small portions of $KMnO_4$ were added while the mixture was stirring at room temperature. After 30 minutes, when the purple color seemed permanent, 30 ml. of water was added and $K_2S_2O_5$ and 5N H_2SO_4 were added to dissolve the MnO_2 and reduce the excess permanganate. The acids were extracted with ether and esterified by refluxing 3 hours with half-saturated methanolic HCl. The yield of esters, 34.3 mg., was 99.5 per cent (calculated for cerebronate).

The chromatographic analysis of these esters is shown in Figure 1 and can be compared with the curve of Figure 2 in the preceding paper (1). The distribution of the various acids is similar in the longer region, but additional peaks have appeared in the shorter region. These arise from overoxidation, presumably by the permanganate; a trial run with synthetic cerebronic acid disclosed that about 7 per cent of the acid was degraded by two carbon atoms instead of one.

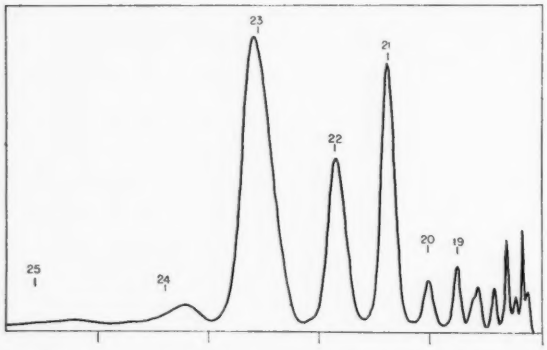

FIG. 1. Gas chromatographic analysis of degradation products of the cerebroside saturated hydroxy acids. The vertical lines under the abscissa represent 10-minute intervals.

This correction was applied to the cerebroside acid degradation products and the mass ratios of the three major esters were found to agree well with the values obtained from the methoxy esters. The ratios for the ethers of the $C_{22, 23, 24}$ esters were 23:16:61; for the degradation products, the ratios were 24:16:61. It may be noted that the retention times for the degradation products matched the expected times particularly well.

The curve for the degradation products quite clearly reveals a longer acid; the corresponding ether peak is more difficult to see. Judging by the retention times in both curves, this trace component is not a straight chain α-hydroxy acid.

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Oxidation of cholesterol by rat liver mitochondria: effect of dietary fat *

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SUMMARY

Rats were maintained on normal diets or on diets containing 20 per cent of corn oil (unsaturated, iodine number 127) or commercial shortening (saturated, iodine number 73) for 40 days. The liver mitochondria of the rats fed saturated fat oxidized cholesterol-26-C^{14} to $C^{14}O_2$ to a much greater extent than did liver mitochondria from rats fed unsaturated fat. In general, liver mitochondria from control rats also oxidized more cholesterol-26-C^{14} than did mitochondria from rats fed unsaturated fat, but this difference was not nearly as consistent (7 of 12 experiments). These results were the same with rats of either sex. Oxidation of sodium pyruvate-2-C^{14} did not vary with diet. The results could not be attributed to differences in liver lipid or cholesterol content. It has been shown that the homologous combination of liver mitochondria and boiled supernatant from the livers of the unsaturated-fat-fed rats greatly impairs cholesterol oxidation. Addition of boiled supernatant from the other dietary groups to liver mitochondria from the rats fed unsaturated fat restores the cholesterol oxidation to normal levels as does substitution of a 10 per cent sucrose solution for the boiled supernatant. Addition of the boiled liver supernatant from the animals fed unsaturated fat to liver mitochondria from the normal or saturated-fat-fed rats has no appreciable inhibitory effect.

The concept that cholesterol is the metabolic precursor of the bile acids has been amply reviewed in the last few years (1, 2, 3). The oxidation of the terminal isopropyl group of the cholesterol side chain has been observed in rat liver slices (4) and in suitably fortified preparations of mouse (5, 6) and rat (7) mitochondria. We have now studied the influence of dietary fats upon the ability of such preparations to oxidize cholesterol and form bile acids. A preliminary report of some of our findings has been given elsewhere (8). Our interest was aroused by several reports that the administration of highly unsaturated fat to humans (9, 10, 11) results in a lowering of serum cholesterol levels and increased fecal excretion of bile acids. In rats (12), increased biliary excretion of bile acids is observed when the rats are fed unsaturated fat.

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† This work was done during the tenure of an Established Investigatorship of the American Heart Association.

METHODS

Wistar rats weighing between 150 and 180 g. were fed rabbit chow [1] which had been thoroughly mixed with 20 per cent fat—either commercial shortening, which is relatively saturated (iodine number 73), or corn oil, which is unsaturated (iodine number 127). The chow was mixed with the added fat until all the fat had been absorbed. These diets will henceforth be designated as "unsaturated" and "saturated." Control animals were maintained on untreated rabbit chow. The rabbit chow used in these experiments contained 2.2 per cent fat (48-hour ether-alcohol extraction), which had an iodine number of 115. It had previously been reported (13) that Purina chow contained 2 per cent fat, iodine number 121, and that 70 per cent of the fatty acids were unsaturated, consisting mostly of linoleic acid. More recently the analysis has been repeated and the new findings are in substantial agreement with the earlier results.[2]

[1] Ralston-Purina Co., St. Louis, Mo.
[2] Personal communication, J. D. Evans.

After 40 days the rats were killed and mitochondrial preparations were made from the livers (7). The livers were removed and placed in 10 per cent aqueous sucrose (w/v). The livers were minced, then homogenized in cold aqueous sucrose (3 volumes of sucrose per volume of minced liver), using a loose-fitting glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at $600 \times g$ for 10 minutes to remove nuclei, unbroken cells, and cellular debris. The supernatant suspension was then centrifuged in a Spinco Model L preparative centrifuge for 12 minutes at $8500 \times g$ to separate mitochondria. The supernatant layer from the second centrifugation was used to prepare the soluble cofactor. The mitochondria were resuspended in 10 per cent sucrose and recentrifuged at $8500 \times g$ for 12 minutes to remove adhering microsomes. The supernatant layer from this centrifugation was discarded. Before use, the mitochondria were resuspended in enough 10 per cent aqueous sucrose to give 1 ml. of suspension per incubation. Generally, the mitochondria obtained from one liver were sufficient for four incubations. The soluble cofactor was prepared by boiling the supernatant from the initial $8500 \times g$ centrifugation. After the boiled solution was freed of denatured protein (filtration or low-speed centrifugation), it was used directly.

Cholesterol-26-C^{14} was synthesized from 3β-hydroxy-Δ^5-norcholesten-25-one by the method of Dauben and Bradlow (14). Solutions of cholesterol were prepared by dissolving cholesterol and Tween 20 (10:1) in a small amount of methanol and, after removal of the methanol in a stream of nitrogen at 40°-50°C, dissolving in the desired buffer. Sodium pyruvate-2-C^{14} was purchased from the Atomic Energy Research Establishment, Amersham, England, and was dissolved directly in the buffer.

Incubations were carried out in stoppered 125 ml. Erlenmeyer flasks containing center wells. The incubation mixture consisted of 1 ml. of the mitochondrial preparation: [3] 1 ml. of a solution containing ATP (25 mg.), DPN (5 mg.), AMP (8 mg.), reduced glutathione (15 mg.), sodium citrate monohydrate (30 mg.), magnesium nitrate hexahydrate (10 mg.), potassium penicillin G (2000 U) and streptomycin sulfate (1 mg.); 5 ml. of labeled substrate in 0.25 M tris (hydroxymethyl) aminomethane.HCl, pH 8.5; and 5 ml. of boiled supernatant. The center well contained 2.0 ml. of 2.5 N sodium hydroxide. The flasks were shaken at 37°C in a water bath for 18 hours. In each experiment cited below, incubations of mitochondria from animals in all three dietary groups were carried out simultaneously. After the incubation period, 2.5 ml. of 25 per cent aqueous trichloroacetic acid was added and the flasks were shaken for 3 hours on a rotary shaker to displace $C^{14}O_2$ from the suspension. The contents of the center well were added to 2.5 ml. of 2 N ammonium chloride and 1 ml. of 1.5 M barium chloride. The resulting barium carbonate precipitate was filtered, weighed, and counted in a Packard Tri-Carb liquid scintillation counter. The barium carbonate was counted as a thixotropic gel using Thixcin [4] as the suspending agent. The method is a modification of the procedure of Funt and Hetherington (15).

For determination of liver lipids, liver samples were thoroughly extracted with petroleum ether, and the extracts were dried and diluted in a volumetric flask. Aliquots were taken for gravimetric determination of total lipid and colorimetric determination of cholesterol. All cholesterol determinations were carried out by the method of Trinder (16). Nitrogen levels of all mitochondrial preparations were determined colorimetrically using Nessler's reagent.

RESULTS AND DISCUSSION

Preliminary experiments were carried out with pooled livers, one male and one female. In view of the findings that male and female rats accumulate liver lipid at different rates (17) and that sex differences exist in the amounts of certain rat-liver enzymes (18), subsequent experiments were carried out with livers from either male or female rats alone. The results (Table 1) show that, regardless of sex, in every case the oxidation of the terminal carbon atoms of cholesterol was greater in liver mitochondrial preparations from rats fed large amounts of saturated fats than in preparations from rats fed equivalent quantities of unsaturated fat. In general, the oxidation of cholesterol by liver mitochondrial preparations from rats on the normal diet was greater than that by mitochondrial preparations from rats fed the unsaturated fat. However, these differences were not as consistent as were those between the oxidative power of the preparations from rats fed saturated and unsaturated fats. The normal rabbit chow contained about 2 per cent of fat with an iodine number of 115. Rats ingesting this diet would be consuming considerably less unsaturated fat than those on the corn oil augmented diet. The variability of the oxidative ability of the preparations most likely reflects differences in activation of this

[3] Abbreviations: ATP, adenosine 5'-triphosphate; DPN, diphosphopyridine nucleotide; AMP, adenosine 5-monophosphate; Tris, tris (hydroxymethyl) aminomethane.HCl; THC, 3α, 7α, 12α-trihydroxycoprostane.

[4] Baker Castor Oil Co., New York, N. Y.

TABLE 1. OXIDATION OF CHOLESTEROL-26-C^{14} BY LIVER MITOCHONDRIA OF RATS *

Sex	Experiment Number	Percentage of Oxidation †		
		Diet		
		Normal	Unsaturated	Saturated
Mixed	1	15.6	1.7	9.6
	2	15.2	17.4	17.6
	3	21.4	3.0	15.5
Females	1	5.3	6.6	15.2
	2	4.2	3.3	12.0
	3	21.0	1.9	10.4
	4	1.3	22.4	31.8
	5	11.7	2.9	4.5
Males	1	7.2	4.9	7.9
	2	15.1	13.9	23.7
	3	15.0	3.0	20.3
	4	11.1	2.8	11.2

* All values corrected for equivalent amounts of mitochondria (mg. N).

† Computed as $BaC^{14}O^3$/cholesterol-26-C^{14}.

mitochondrial oxidase system. The system has been shown to be sensitive to many minor variations in preparation or technique (7). The consistency of our findings in the face of this sensitivity is worth noting.

When incubations were conducted with boiled mitochondria for periods up to 22 hours, there was no oxidation of cholesterol and no production of radioactive carbon dioxide. This finding, plus the presence of two antibiotics in all incubations, would appear to exclude bacterial contamination as the source of cholesterol oxidase activity. In one time-course experiment several preparations from the same liver were incubated for 2, 4, 6, and 18 hours. A gradual oxidation with time is noted for all three preparations (Fig. 1).

Oxidation of Other Substrates. The effect of these diets on mitochondrial oxidation of substrates other than cholesterol was also of interest, and several experiments were carried out in which the substrate was sodium pyruvate-2-C^{14}. In general, there was little effect of diet, as can be seen from Table 2. The limited extent of pyruvate oxidation as compared with cholesterol oxidation may be largely attributable to the relatively high pH (8.5) at which these incubations were carried out. Relative thiamine deficiency of the mitochondria due to extraction by the aqueous sucrose

TABLE 2. OXIDATION OF PYRUVATE-2-C^{14} BY LIVER MITOCHONDRIA OF RATS AT pH 8.5 *

Sex	Diet	Percentage of Oxidation †	
		Experiment 1	Experiment 2
M	normal	0.6	2.0
M	unsaturated	0.4	0.7
M	saturated	0.9	0.6
F	normal	14.4	3.6
F	unsaturated	6.4	4.1
F	saturated	7.5	3.9

* All values corrected for equivalent amounts of mitochondria (mg. N).

† Computed as $BaC^{14}O_3$/sodium pyruvate-2-C^{14}.

solution may also contribute to this discrepancy. In one series in which the substrate was 3α, 7α, 12α-trihydroxycoprostane-26-C^{14} (19), the extent of oxidation was 42.8, 46.0, and 75.4 per cent for the normal, unsaturated, and saturated mitochondrial preparations, respectively.

Analysis of Liver Lipids. The finding of differences in oxidation rates associated with dietary differences raises the question of the amount of fat being deposited in the liver and its possible effect on the mitochondria. Some autopsy data are given in Table 3. Several other studies have been concerned with the liver weights, liver lipids, and serum cholesterol levels

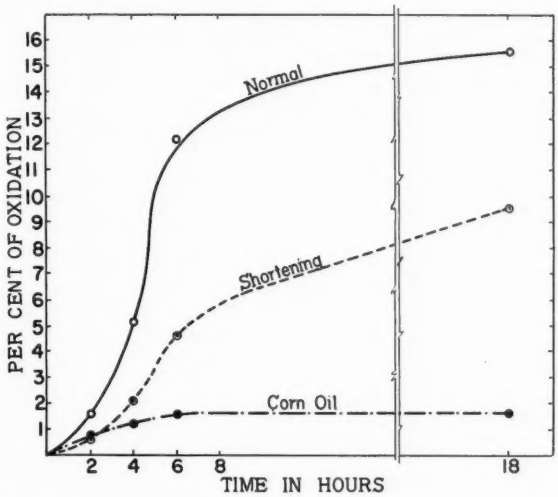

FIG. 1. Time-course of oxidation of cholesterol-26-C^{14} by liver mitochondria.

TABLE 3. AUTOPSY DATA ON RATS FED SATURATED AND UNSATURATED FAT
(Average of 12 Rats per Group)

Sex	Diet	Avg. Body Wt.	Avg. Liver Wet Wt.	Liver Wt. as Percentage of Body Wt.	Serum Cholesterol
		gm.	*gm.*		*mg./100 ml.*
M	normal	258	9.00	3.45	104.4 ± 9.9 *
M	unsaturated	259	8.58	3.31	77.2 ± 23.1
M	saturated	230	7.11	3.09	110.0 ± 5.9
F	normal	206	7.21	3.50	78.9 ± 20.5
F	unsaturated	200	6.78	3.39	82.4 ± 10.7
F	saturated	192	6.83	3.56	110.0 ± 16.0

* Standard deviation.

of rats maintained on diets containing saturated and unsaturated fat (20, 21, 22). These investigators have also found lower serum cholesterol levels in animals fed unsaturated fat, and larger livers in the males. Our data also show that the males had larger livers; however, we found that the ratio of liver weight to body weight was greater in the females. Analysis of the liver lipid and cholesterol content shows slightly more fat in the female livers than in the males, and in both sexes more liver lipid in the groups on the unsaturated fat diets. Our data are presented in Table 4.

The lower fat content of the livers of the rats fed saturated fat might possibly affect the oxidative ability of the mitochondrial preparations if the amount of lipid in the mitochondria reflected the over-all differences in liver lipid.

The amounts of lipid found in the livers of animals fed saturated and unsaturated diets were closer to the normal than has been reported by the other workers cited. However, in these other studies the fats compared were cottonseed oil and either lard or coconut oil, both of which differ in composition and extent of saturation from the saturated fat we have used. One experiment, in which corn oil was compared with margarine, among other fats, showed essentially similar levels of liver fat (23). All the other work was done with formula diets.

The question remained whether the differences in liver lipid were reflected in the mitochondrial lipid content. Previous analyses of liver fractions have shown most of the cholesterol to be present in a fraction sedimenting at 105,000 × *g* (24). The mitochondria contained about 0.2 mg. of cholesterol per 4 ml. (25). In these experiments the mitochondria were sedimented by centrifugation at 24,000 × *g* for 10 minutes. Our own analyses of liver mitochondria (sedimented at 8500 × *g* for 12 minutes) showed that about 0.10 mg. of cholesterol per ml. was present in the mitochondria taken from livers of rats of both sexes on all three diets. The mitochondrial lipid content was similar in all groups of rats.

Rat mitochondria have been shown to contain almost no esterified cholesterol (25). To test possible effects of transesterification, the incubation mixtures were subjected to extraction with ether and petroleum ether and, after addition of carrier cholesterol, the free cholesterol was precipitated as the digitonide. The supernatant fluid was saponified, carrier cholesterol was added, and digitonin precipitation was repeated. All of the radioactivity was present in the first (free cholesterol) digitonide.

To ascertain the possible effects of the liver lipids on cholesterol oxidation, pooled livers from rats (one male and one female) fed each of the three diets were extracted with ether-alcohol (1:3). The extracts were dried over anhydrous sodium sulfate and all the solvent removed at reduced pressure. Weighed amounts of the extracted liver fat from all three preparations were emulsified according to the method used for the labeled substrates, using Tween 20 and Tris buffer. Solutions of the fat from each preparation (1 mg.) were added to normal male and female rat liver mitochondrial preparations. The extent of inhibition of oxidation of cholesterol-26-C^{14} by each type of liver fat was of the same order of magnitude (Table 5). In experiments using sodium pyruvate-2-C^{14} as the substrate, we again observed similar inhibitory effects of all three fat preparations in the male series, while in the females there was no inhibition when fat from normal livers was added. These data are also presented in Table 5.

"Crossover" Experiments. In another effort to eluci-

date the source of the observed differences in cholesterol oxidation, we added supernatant fractions of one preparation to the mitochondrial preparation of another. These "crossover" experiments were carried out with both cholesterol-26-C^{14} and sodium pyruvate-2-C^{14} as substrates. In all, six such experiments were carried out with cholesterol and two with pyruvate. The results for cholesterol were in general agreement in all cases, regardless of sex. They showed that the addition of "foreign" supernatant or of 10 per cent aqueous sucrose to liver mitochondria from controls or saturated fat-fed animals resulted in roughly the same extent of oxidation as did the addition of homologous supernatant fractions. There was considerable enhancement of oxidation by liver mitochondria from animals fed the unsaturated fat when either aqueous sucrose or the supernatant fractions from livers of the control rats or saturated-fat-fed rats were substituted for the homologous supernatant fraction. A representation of the average effects may be found in Table 6. In view of the variability of the oxidative power of individual preparations, any differences of less than 100 per cent may be considered within the range of variability. Whether the oxidative activity of the mitochondria alone or of a homologous combination of mitochondrial preparation and supernatant be taken as the reference, it is apparent that the combination of mitochondria from the livers of animals fed unsaturated fat and the homologous supernatant has far less oxidative activity than the mitochondria alone. The results of adding the supernatant from the livers of animals fed unsaturated fat to liver mitochondria prepared from normal or saturated-fat-fed animals indicate that the "unsaturated" supernatant exerts no noxious effect per se. The cause of the observed inhibition in the homologous unsaturated preparation is under investigation. The data from the crossover experiments with sodium pyruvate-2-C^{14} are given in Table 7. Very little difference is seen between the various pyruvate incubations.

TABLE 4. LIVER LIPIDS OF RATS FED SATURATED AND UNSATURATED FAT
(Average of 5 Rats per Group)

Sex	Diet	Percentage of Liver Lipid	Percentage of Liver Cholesterol
M	normal	3.5 ± 0.95 *	0.38 ± 0.05 *
M	unsaturated	4.0 ± 1.65	0.46 ± 0.18
M	saturated	3.7 ± 0.80	0.34 ± 0.06
F	normal	3.7 ± 1.07	0.35 ± 0.15
F	unsaturated	4.8 ± 1.36	0.45 ± 0.10
F	saturated	4.2 ± 0.19	0.44 ± 0.12

* Standard deviation.

GENERAL COMMENTS

Fredrickson (26, 27) has demonstrated that cholesterol may be oxidized by mouse liver preparations to products resembling, but not identical with, deoxycholic and cholic acids. The acidic fractions obtained from experiments in which cholesterol-4-C^{14} had been incubated with rat liver mitochondrial preparations from all dietary groups were subjected to paper

TABLE 5. INHIBITION OF OXIDATION OF CHOLESTEROL-26-C^{14} AND PYRUVATE-2-C^{14} BY LIVER FAT FROM RATS ON VARIOUS DIETS

Sex	Added Fat	Cholesterol-26-C^{14}		Pyruvate-2-C^{14}	
		Percentage of Oxidation *	Percentage of Inhibition †	Percentage of Oxidation *	Percentage of Inhibition †
M	normal	1.8	54	4.5	25
M	unsaturated	2.0	50	4.2	30
M	saturated	1.4	64	4.2	30
M	——	3.9	——	6.0	——
F	normal	1.5	69	3.2	0
F	unsaturated	1.8	63	2.4	25
F	saturated	1.9	61	2.1	14
F	——	4.8	——	2.8	——

* All values corrected for equivalent amounts of mitochondria (mg. N).
† Inhibition based on control.

TABLE 6. EFFECT OF DIFFERENT SUPERNATANT FRACTIONS ON OXIDATION OF CHOLESTEROL-26-C^{14} BY RAT LIVER MITOCHONDRIA (Average of 6 Experiments)

Mitochondria	Supernatant	Effect on Oxidation	
		Homologous = 1 †	No Supernatant = 1 ‡
normal	normal	1.0	1.4 ± 0.3
normal	unsaturated	1.0 ± 0.1 §	1.5 ± 0.4
normal	saturated	1.3 ± 0.1	1.8 ± 0.4
normal	——*	0.7 ± 0.1	1.0
unsaturated	normal	4.2 ± 1.7	1.3 ± 1.0
unsaturated	unsaturated	1.0	0.3 ± 0.2
unsaturated	saturated	2.3 ± 1.1	0.7 ± 0.3
unsaturated	——	3.2 ± 2.1	1.0
saturated	normal	1.1 ± 0.3	1.1 ± 0.2
saturated	unsaturated	1.0 ± 0.1	1.1 ± 0.1
saturated	saturated	1.0	1.0 ± 0.1
saturated	——	1.0 ± 0.3	1.0

* 10% sucrose used instead of boiled supernatant.
† Ratio of oxidative ability if oxidation by homologous mitochondria and supernatant = 1.
‡ Ratio if mitochondria plus sucrose = 1.
§ Standard error.

TABLE 7. EFFECT OF DIFFERENT SUPERNATANT FRACTIONS ON OXIDATION OF SODIUM PYRUVATE-2-C^{14} AT pH 8.5 BY RAT LIVER MITOCHONDRIA

Mitochondria	Supernatant	Percentage of Oxidation	
		Experiment 1 (Males)	Experiment 2 (Females)
normal	normal	0.6	3.6
normal	unsaturated	0.4	1.8
normal	saturated	0.6	3.6
normal	——	0.4	3.0
unsaturated	normal	0.5	4.2
unsaturated	unsaturated	0.4	4.1
unsaturated	saturated	0.4	6.2
unsaturated	——	0.3	1.8
saturated	normal	0.8	2.3
saturated	unsaturated	0.9	3.8
saturated	saturated	1.0	3.9
saturated	——	0.9	2.4

chromatography in two different solvent systems (28, 29). In all cases the bulk of the radioactivity (assayed by scanning) was found at an R_f approximating that of a known sample of cholic acid detected with antimony trichloride (30).

Since the early reports that human serum cholesterol levels could be lowered by ingestion of unsaturated fat (31, 32), the effect of this fat on cholesterol metabolism has been the subject of considerable speculation. Data suggesting that the mechanism of serum cholesterol lowering involves increased fecal excretion of bile acids have been cited (9 to 12). Apparently the increased excretion of bile acid and lowering of serum cholesterol levels observed during intake of unsaturated fat take place despite increased cholesterol biosynthesis (33, 34, 35) and, as these studies show, decreased cholesterol oxidation. Our animals showed a relative hypocholesterolemia when fed unsaturated fat despite increased hepatic synthesis (inferred from references 33, 34, 35) and decreased hepatic oxidation. Unsaturated fat has also been shown to enhance intestinal absorption of cholesterol (12). It has been found that decreased reabsorption of bile acid results in a decrease of plasma cholesterol levels (36). Reduced bile acid production may have a similar effect. Perhaps the increased oxidation of cholesterol observed on the saturated-fat diet may result in elevated levels of circulating bile acids or bile salts which may, in turn, have a hypercholesterolemic effect such as is observed when bile acids or salts are fed to rats (37, 38) or rabbits (39). These speculations must await experimental testing.

Our data are sustained to some extent by the findings of Wilson and Siperstein (40), that rats fed saturated fat (lard) over long periods of time, when given a single tracer dose of cholesterol-4-C^{14}, excrete more radioactivity as bile acid than do rats maintained on corn oil or fat-free diets for similar lengths of time.

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Metabolism of adipose tissue: incorporation of isoleucine carbon into lipids by slices of adipose tissue*

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SUMMARY

Slices from adipose tissue and liver were incubated with uniformly labeled C^{14}-isoleucine and the respiratory carbon dioxide, fatty acids, and nonsaponifiable lipids were isolated and analyzed for radioactivity. Organic acids were qualitatively identified by chromatography and autoradiography. Adipose tissue oxidized isoleucine to carbon dioxide at a rate greater than that of liver. Conversion to nonsaponifiable lipids was small for both tissues. Recovery of C^{14} from isoleucine C^{14} in fatty acids of adipose tissue was 50 to 100 times greater than recovery in liver slices. C^{14} was recovered in acetate, propionate, methylmalonate, and α-methylacetoacetate in adipose tissue after incubation with isoleucine. The role of acetate, propionate, methylmalonate, α-methylacetoacetate, and isoleucine in biosynthesis of fatty acids in adipose tissue is discussed.

Adipose tissue is now considered to be a metabolically active tissue containing most of the enzymes required to carry on reactions common to other mammalian tissues. Thus, many of the schemes that have been described for utilization, storage, and interconversion of carbohydrates and fats in muscle, liver, and kidney have also been described in adipose tissue. However, the quantitative differences previously observed (1, 2) for the conversion of certain metabolites to long-chain fatty acids suggest that adipose tissue contains a pathway involving branch-chain fatty acids, not found in other tissues.

Propionate, for instance, is believed to be converted to fatty acids by a path that includes methylmalonate as an intermediate:

$$\text{propionate} + (X) \rightarrow \text{methylmalonate} \rightarrow \text{fatty acids.}$$

When C^{14}-bicarbonate was tested as a possible (X) in slices of adipose tissue, no radiocarbon appeared in the synthesized fatty acids (2).

A possible role for isoleucine in connection with these studies was suggested by the work of Coon and co-workers (3). These investigators have shown that isoleucine is degraded in rat liver slices to propionate and other compounds by way of a series of reversible reactions. In order to understand the nature of this novel pathway for the biosynthesis of long-chain fatty acids, the present investigation was undertaken on the metabolism of isoleucine in adipose tissue.

* Aided by grants from the American Cancer Society, the Schering Corporation, and Grant A-2174 from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

EXPERIMENTAL

Radioactive Substrates. Uniformly labeled C^{14}-L-isoleucine with a specific activity of 7.35 mc. per mmole was purchased from Nuclear-Chicago Corporation.

Preparation and Treatment of Slices. Male white Swiss mice were fed *ad libitium* an adequate diet of Purina laboratory chow until the time of sacrifice. The adult mice were killed by separation of the cervical vertebrae, and the liver and adipose tissue were rapidly removed. The preparation of slices and incubation procedures were identical with those employed in our previous investigations (1, 2). Further experimental conditions are described in the Tables.

Analytical Procedures. Collection of CO_2, separation and isolation of lipid fractions, and radioassay methods have been described elsewhere (1, 2). After collection of CO_2, the slices and medium were transferred to a tube and centrifuged. The supernatant fluid was decanted. The tissue was extracted twice with 5 ml. of hot water, and the combined supernatant fraction was treated for chromatography as described below.

Hydrazone derivatives of keto-acids were prepared by grinding the tissue in a Potter-Elevehjem homogenizer in the presence of 2,4-dinitrophenylhydrazine (4). Five mg. of the hydrazine dissolved in 1 ml. of 6 N HCl was used per gram of tissue. The suspension was allowed to stand for 15 minutes and cell debris was separated by centrifugation. The aqueous solution was extracted with three, 15 ml. volumes of a chloroform-ethanol (80:20, v/v) mixture. The hydrazones were then extracted from this mixture with 15 ml. of 1 N Na_2CO_3 and the chloroform-ethanol layer was discarded. The Na_2CO_3 solution was washed with 10 ml. of chloroform-ethanol to remove any unchanged hydrazine. The Na_2CO_3 solution was acidified with 6 N HCl and the hydrazones extracted with three, 10 ml. volumes of chloroform-ethanol. The organic phase was then dried under a gentle stream of air. The hydrazones were separated and identified by paper chromatography as described below.

Radiochromatography. Procedures for identifying methylmalonic acid were described previously (2). Acetic and propionic acids were converted to their ammonium salts and chromatographed with 95 per cent ethanol—NH_4OH (100:1, v/v) as described by Kennedy and Barker (5). The hydrazones of the α-keto acids were dissolved in a minimum amount of 0.1 M glycine-NaOH solution, pH 7.4 and streaked on Whatman No. 2 paper, which had been impregnated previously with glycine solution and dried. The

TABLE 1. CONVERSION OF C^{14}-ISOLEUCINE TO FATTY ACIDS, NONSAPONIFIABLE LIPIDS, AND CARBON DIOXIDE BY SLICES OF ADIPOSE TISSUE AND LIVER*

Experiment	Tissue	C^{14} Recovered as			C^{14} per 100 mg. Fat-free Tissue Recovered as		
		Fatty Acids	Non-saponifiable Lipids	CO_2	Fatty Acids	Nonsaponifiable Lipids	CO_2
		total cpm.	*total cpm.*	*total cpm.*	*percentage*	*percentage*	*percentage*
1	Adipose	191,000	2,180	193,000	1.11	0.01	1.09
2	Adipose	83,000	2,910	103,000	0.38	0.01	0.46
3	Adipose	239,000	2,920	166,000	1.24	0.02	0.86
4	Adipose	94,000	2,730	216,000	0.50	0.01	1.15
5	Adipose	111,000	4,800	186,000	0.49	0.02	0.82
Mean					0.74 ± 0.16 †	0.01 ± 0.00	0.88 ± 0.11
6	Liver	6,720	5,450	141,000	0.01	0.01	0.24
7	Liver	5,280	7,020	145,000	0.01	0.01	0.24
8	Liver	4,730	4,210	117,000	0.01	0.01	0.20
9	Liver	9,010	7,510	122,000	0.02	0.01	0.26
10	Liver	7,500	5,960	120,000	0.01	0.01	0.19
Mean					0.01 ± 0.00	0.01 ± 0.00	0.23 ± 0.01

* 1460 ± 20 mg. of tissue slices were incubated for 3 hours at 37.5°C with 0.001 M C^{14}-isoleucine. The total activity added to each flask was 4.5×10^6 cpm. 14.0 ml. of Krebs-bicarbonate buffer enriched with 0.01 M succinate and 0.011 M glucose was used.

† Standard error of the mean.

TABLE 2. EFFECT OF PROPIONATE ON CONVERSION OF C^{14}-ISOLEUCINE TO FATTY ACIDS, NONSAPONIFIABLE LIPIDS, AND CARBON DIOXIDE BY SLICES OF ADIPOSE TISSUE AND LIVER *

Experiment	Tissue	C^{14} Recovered as			C^{14} per 100 mg. Fat-free Tissue Recovered as		
		Fatty Acids	Nonsaponifiable Lipids	CO_2	Fatty Acids	Nonsaponifiable Lipids	CO_2
		total cpm.	*total cpm.*	*total cpm.*	*percentage*	*percentage*	*percentage*
1	Adipose	2,820	240	6,920	0.08	0.01	0.21
2	Adipose	2,660	100	6,720	0.06	<0.01	0.16
3	Adipose	2,570	240	3,600	0.06	0.01	0.09
Mean					0.07 ± 0.01 †		0.15 ± 0.03
4	Liver	920	310	2,150	0.01	<0.01	0.02
5	Liver	830	300	6,460	0.01	<0.01	0.07
6	Liver	620	720	5,190	0.01	0.01	0.06
Mean					0.01 ± 0.00		0.05 ± 0.01

* 1430 ± 10 mg. of tissue slices were incubated for 3 hours at 37.5°C with 0.001 M C^{14}-isoleucine. The total activity added to each flask was 700,000 cpm. 14.0 ml. of Krebs-bicarbonate buffer enriched with 0.011 M glucose was used. Propionate was also added to a final concentration of 0.01 M.

† Standard error of the mean.

chromatogram was developed in tertiary amyl alcohol:ethanol:water (50:10:40, v/v/v) for 48 hours by the ascending method.

"No screen" X-ray films were used for the preparation of autoradiograms.

RESULTS

C^{14}-isoleucine Experiment. Isoleucine was readily metabolized by adipose tissue as shown by the data in Table 1. This tissue converted isoleucine to long-chain fatty acids in amounts comparable to those observed previously for acetate, propionate, and methylmalonate; a mean value of 0.74 per cent per 100 mg. of fat-free tissue was recovered as C^{14}-labeled fatty acids after incubation with C^{14}-isoleucine. This may be compared to values of 1.60 per cent for propionate-1-C^{14}, 1.25 per cent for acetate-2-C^{14} and 0.52 per cent for methylmalonate (1, 2). When compared to liver, the extent of conversion of isoleucine to long-chain fatty acids was 50 to 100 times greater in favor of adipose tissue.

Adipose tissue was also able to oxidize the isoleucine to CO_2 to a greater extent than liver: 0.88 per cent per 100 mg. fat-free tissue was the average value found for conversion of isoleucine to CO_2 by adipose tissue compared to 0.23 per cent for liver.

Small but detectable amounts of isoleucine were converted to nonsaponifiable lipids by both tissues.

C^{14}-isoleucine Carrier Propionate Experiment. Addition of carrier propionate or methylmalonate to C^{14}-isoleucine was next tested to determine whether these compounds could serve as trapping agents to depress conversion of isoleucine to fatty acids.

Addition of carrier propionate to a final concentration of 0.01 M reduced the conversion of C^{14}-isoleucine to fatty acids by adipose tissue from a value of 0.74 per cent (Table 1) to a value of 0.07 per cent (Table 2). A somewhat less marked depression of about sixfold was also noted in the conversion of isoleucine to carbon dioxide. In the case of liver slices, addition of carrier propionate depressed the conversion of isoleucine to carbon dioxide from a value of

0.23 per cent to 0.05 per cent per mg. fat-free tissue. There was no corresponding depression in conversion to fatty acids.

C^{14}-isoleucine Carrier Methylmalonate Experiment. Addition of carrier methylmalonate also depressed the conversion of isoleucine to fatty acids in adipose tissue (Table 3), although to a lesser degree than that observed by the addition of carrier propionate. The value was found to be 0.38 per cent of added C^{14}, converted to fatty acids by adipose tissue with carrier methylmalonate as compared to a value of 0.74 per cent without added carrier. Only a slight depression in conversion of isoleucine to carbon dioxide with added methylmalonate was found (Table 3).

A possible explanation for the greater inhibition brought about by the addition of carrier propionate as compared to methylmalonate would be that the former penetrated the cell at a much faster rate than the latter. Or perhaps methylmalonate was converted to an activated form, probably the acyl coenzyme A derivative, at a much slower rate than propionate. A comparison of the rates of oxidation of these compounds (propionate-1-C^{14}, 6.90 per cent and methylmalonate with carboxyl-C^{14}, 0.08 per cent) renders either of these explanations feasible.

Addition of carrier methylmalonate caused no depression in conversion of isoleucine to fatty acid in liver. A slight depression in conversion to carbon dioxide was observed for this tissue (Tables 1 and 3).

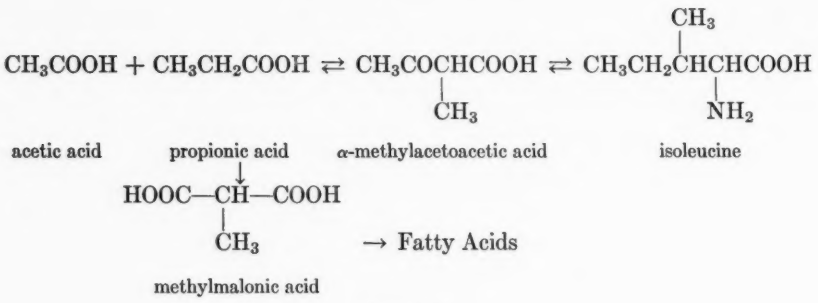
Chromatography-Autoradiography. Figure 1 shows recovery of C^{14} in the methylmalonate spot after incubation of adipose and liver tissues with C^{14}-isoleucine. No radioactivity could be detected in the area where succinate would be expected to migrate. Figure 2 shows recovery of C^{14} in acetate and propionate spots for both tissues. α-Methylacetoacetate containing radiocarbon was found after incubation with isoleucine, as shown in the radiogram in Figure 3.

TABLE 3. EFFECT OF METHYLMALONATE ON CONVERSION OF C^{14}-ISOLEUCINE TO FATTY ACIDS, NONSAPONIFIABLE LIPIDS, AND CARBON DIOXIDE BY SLICES OF ADIPOSE TISSUE AND LIVER*

Experiment	Tissue	C^{14} Recovered as			C^{14} per 100 mg. Fat-free Tissue Recovered as		
		Fatty Acids	Nonsaponifiable Lipids	CO_2	Fatty Acids	Nonsaponifiable Lipids	CO_2
		total cpm.	*total cpm.*	*total cpm.*	*percentage*	*percentage*	*percentage*
1	Adipose	5,630	410	9,230	0.23	0.01	0.38
2	Adipose	6,030	190	8,620	0.28	0.01	0.39
3	Adipose	9,820	210	13,900	0.44	0.01	0.62
4	Adipose	6,320	130	10,400	0.27	0.01	0.63
5	Adipose	16,400	320	22,800	0.70	0.02	0.98
Mean					0.38 ± 0.08 †	0.01 ± 0.00	0.60 ± 0.10
6	Liver	800	600	8,190	0.01	0.01	0.14
7	Liver	1,230	380	8,850	0.02	0.01	0.15
8	Liver	390	320	9,700	0.01	0.01	0.19
9	Liver	310	420	7,250	0.01	0.01	0.13
10	Liver	410	610	7,420	0.01	0.01	0.13
Mean					0.01 ± 0.00	0.01 ± 0.00	0.15 ± 0.01

* 1420 ± 10 mg. of tissue slices were incubated for 3 hours at 37.5°C with 0.001 M C^{14}-isoleucine. The total activity added to each flask was 43,000 cpm. 14.0 ml. of Krebs-bicarbonate enriched with 0.01 M succinate and 0.011 M glucose was used. Methylmalonate was also added to a final concentration of 0.01 M.

† Standard error of the mean.



SCHEME 1

DISCUSSION

In a previous report (2) it was suggested that propionate might first be converted to methylmalonate en route to the formation of long-chain fatty acids in adipose tissue. Further evidence supporting this scheme has since been reported (6). C^{14}-methylmalonate has been recovered following addition of propionate-1-C^{14} to slices of adipose tissue. Methylmalonate is formed by a mechanism not utilizing condensation of bicarbonate with propionate as a major role. However, the recovery of C^{14}-succinate following incubation of C^{14}-bicarbonate with carrier propionate in slices of adipose tissue (2) indicates that the scheme described by Flavin and Ochoa for methylmalonate synthesis must still be considered (7).

These results raised the possibility that propionate condenses with a compound of chain length greater than 1 carbon in the formation of intermediates that are converted first to methylmalonate and secondly to long-chain fatty acids. Isoleucine was tested as a possible precursor in this pathway, since this compound has been shown by Coon and associates (3) to be degraded to a five-carbon keto-acid, α-methylacetoacetate, which in turn is in equilibrium with propionate and acetate.

Recovery of radioactivity in spots corresponding to acetate, propionate, and α-methylacetoacetate after incubation with C^{14}-isoleucine indicates that the series of reactions outlined by Coon *et al.* for other tissues likewise is operative in adipose tissue. The depression in conversion of isoleucine to fatty acids in adipose tissue after addition of high concentrations of carrier propionate supports the theory that isoleucine is degraded to this compound or to an intermediate that is in equilibrium with it.

These results are consistent with two possible schemes for describing a path that may play a major role in the over-all synthesis of lipids in adipose tissue. One possible scheme would be as shown in Scheme 1 (centered above), where isoleucine is first degraded to propionate, which in turn is converted to lipids. Methylmalonate is an intermediate in the last step.

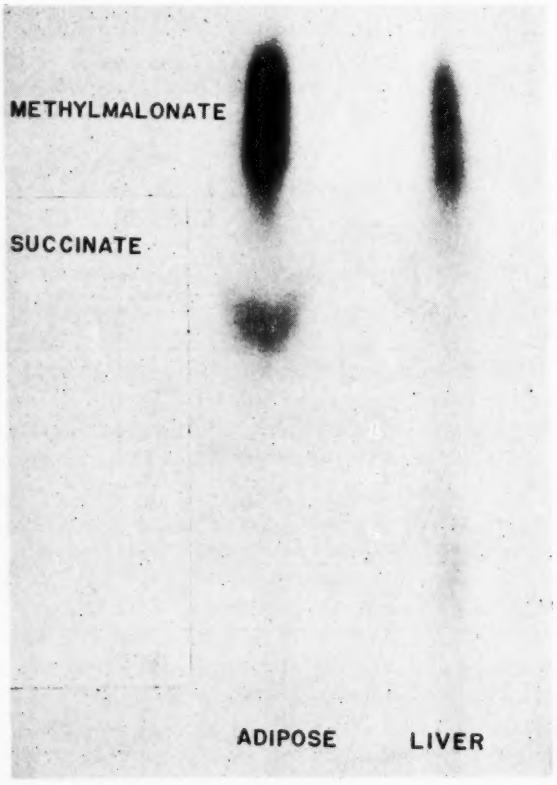
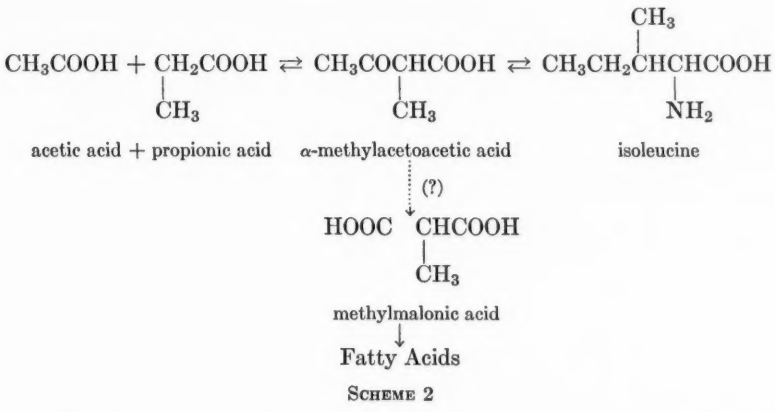


FIG. 1. Autoradiograms of adipose tissue and liver extracts from mice. Supernatant and combined washings were electrodialyzed, extracted with ether, and chromatographed for 20 hours with isoamyl alcohol-4 N formic acid. Tissues were incubated with C^{14}-isoleucine. R_f values obtained for controls at the points indicated are as follows: methylmalonate, 0.85 and succinate, 0.74.



SCHEME 2

In this scheme isoleucine conversion to fatty acids would be depressed by the addition of carrier propionate and methylmalonate, which fits the facts.

Another scheme, which is purely hypothetical, would be as shown in Scheme 2 (centered above). This scheme is consistent with the result obtained in this paper. Furthermore, it explains why carbon-1 of acetate is incorporated to a greater extent than carbon-2 by slices of adipose tissue, results which were obtained and reported earlier (1).

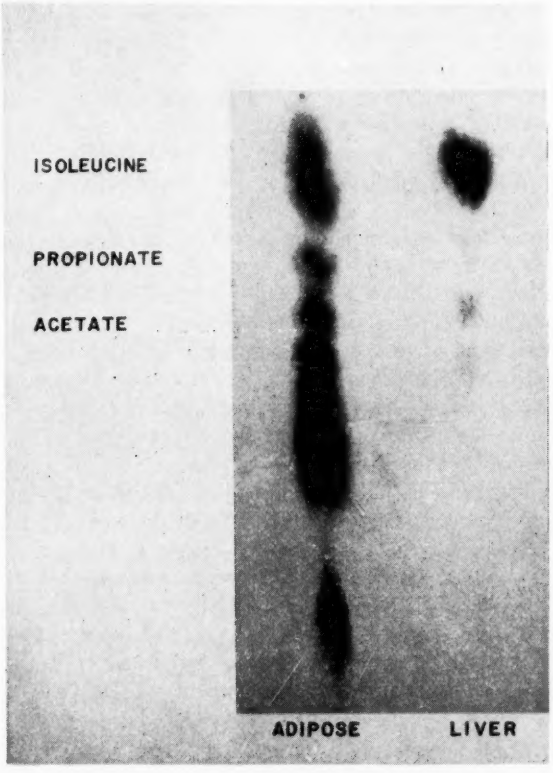


FIG. 2. Autoradiograms of adipose tissue and liver extracts from mice prepared as in Figure 1. The ammonium salts of the organic acids were chromatographed for 20 hours with ethanol—NH_4OH—H_2O. Tissues were incubated with C^{14}-isoleucine. R_f values obtained for controls at the points indicated are as follows: isoleucine, 0.58, propionate, 0.46, and acetate, 0.38.

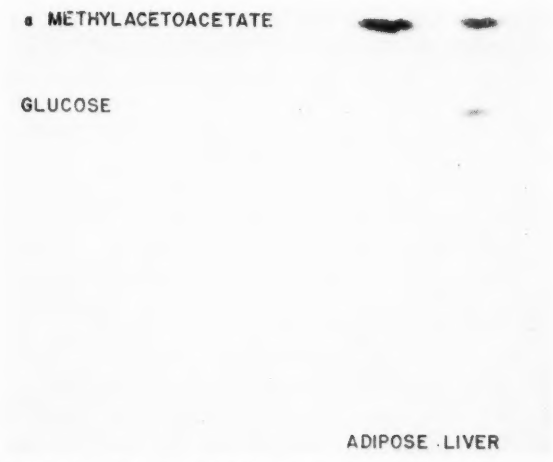


FIG. 3. Autoradiograms of hydrazones from adipose tissue and liver. Tissues were ground in a solution of 2,4-dinitrophenylhydrazine. After purification, the hydrazones were chromatographed for 48 hours with tertiary amyl alcohol-ethanol-water. Tissues were incubated with C^{14}-isoleucine. R_f values obtained for controls at the points indicated are as follows: α-methylacetoacetate, 0.96 and glucose, 0.78.

For the sake of brevity, the schemes do not include all the intermediates that would be expected to intervene between the compounds indicated, nor do they show the compounds in their possible activated forms. Other established paths for lipid synthesis, such as condensation of two acetate molecules, are omitted.

Acetate, propionate, methylmalonate, and isoleucine have all been shown to be very active in their ability to form long-chain fatty acids. The last three substrates, in fact, have been shown to form fatty acids much more readily in adipose tissue than in liver. Thus the data suggest that adipose tissue might have a specific function in synthesizing fats from these metabolites and that the scheme shown above might play a major role in this activity. Investigation concerning the mechanism and intermediates in the pathway as well as the nature of the long-chain fatty acids formed are the subject of further study.

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Volume 1
Number 1

The liberation of lipoprotein lipase by heparin from adipose tissue incubated *in vitro*

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SUMMARY

Epididymal fat tissues of rats, incubated *in vitro* in the presence of heparin, have been found to release lipoprotein lipase into the medium. This release occurs rapidly and shows little dependence upon the composition of the medium. The tissues of carbohydrate-fed rats are more active with respect to lipoprotein lipase liberation than those of fasted animals.

The administration of heparin parenterally to animals or man has been shown to cause the appearance in plasma of a "clearing factor" with the ability to cause the clearing of lactescence (1, 2); heparin itself added to plasma *in vitro* is without effect. This factor has been characterized by Korn (3, 4) and Korn and Quigley (5, 6) and renamed lipoprotein lipase, an enzyme which has a specificity for hydrolysis of triglycerides contained in soluble lipoproteins. Free fatty acids liberated by this reaction have been shown to inhibit the lipase activity and an acceptor such as serum albumin is needed for the reaction to proceed (7). Methods for the assay of biological preparations for lipoprotein lipase activity have been standardized by Korn (8), and depend upon the measurement of hydrolysis of a triglyceride substrate.

The presence of lipoprotein lipase in extracts prepared from rat and chicken adipose tissue has been shown by Korn and Quigley (5, 6). That peripheral tissues release lipoprotein lipase on perfusion with heparin-containing blood was shown by Jeffries (9); it therefore seemed possible that adipose tissue might be an important source of lipoprotein lipase in the intact animal. The present experiments were undertaken to determine the ability of heparin to elicit the release of lipoprotein lipase from surviving adipose tissue incubated *in vitro*.

METHODS

The animals used in these experiments were young male rats of the Sprague-Dawley strain, weighing from 150 to 220 g. They were sacrificed by an abrupt blow on the head, and the two epididymal fat bodies were quickly excised. These tissues were placed in 25 ml. Erlenmeyer flasks, each containing approximately 1 ml. of Krebs-Ringer phosphate buffer per 100 mg. wet weight of tissue, and incubated at 37°C with gentle agitation in a Dubnoff metabolic shaking incubator. Heparin, albumin, and other agents were added to the incubation system of one epididymal fat body from each rat, as indicated in the descriptions of the individual experiments; the contralateral tissue served as a control. The lipase activity of an aliquot of each incubation medium was assayed for its ability to produce free fatty acids from a coconut oil substrate; one unit of lipoprotein lipase was considered to be that amount of lipase which would release one μmole of fatty acid per hour. The substrate was prepared by mixing 8 parts of a 10 per cent solution of bovine serum albumin (Armour Fraction V), adjusted to pH 8.7 by titration with ammonium hydroxide, 1 part of a 4 per cent coconut oil emulsion prepared by dilution of Ediol® [1] with water, and 1 part of fresh pooled human serum, and incubating the mixture for 30 minutes at 37°C. An aliquot (0.5 ml.) of the solution to be assayed for lipoprotein lipase was mixed with 0.5 ml. of this substrate (giving a final pH of 8.5), and incubated for one hour at 37°C. The free fatty acids evolved were determined by the method of Dole (10), modified to the extent that Nile blue A was used in place of thymol blue for the titrations. The use of a glass-stoppered centrifuge tube for the enzyme-substrate mixture during the lipase assay made it possible to extract the free fatty acids without further transfer of the sample. The addition of the isopropanol-heptane extraction mixture served as a conven-

[1] Ediol (Schenley Laboratories, Lawrenceburg, Ind.) is an aqueous fat emulsion containing 50 per cent coconut oil, 12.5 per cent sucrose, 1.5 per cent glyceryl monostearate, and 2 per cent polyoxyethylene sorbitan monostearate.

ient means of terminating the lipolytic reaction. Preliminary experiments indicated that under these conditions free fatty acid evolution proceeded at a constant rate for 2 hours, and was a linear function of the enzyme concentration.

RESULTS

Rate of Release of Lipase in the Presence of Heparin. The incubation of tissue in the presence of heparin was accompanied by the rapid release of lipase into the medium. In order to follow the time-course of enzyme liberation, an experiment was performed in which epididymal fat bodies (one from each of six fed rats) were pooled and incubated in 30 ml. of Krebs-Ringer phosphate buffer, to which had been added sodium heparin (128 units per mg., U.S.P.) in a concentration of 30 μg. per ml. The six contralateral tissues were incubated under identical conditions but for the omission of heparin. At the intervals plotted in Figure 1, 1.0 ml. of each medium was withdrawn for duplicate lipase assays; the volume of medium was not replaced. Although the medium in which the tissues were incubated without heparin did not show measurable lipolytic activity, lipase was found in the heparin-containing medium in as little as 2 minutes. As may be seen in Figure 1, this activity increased rapidly and then declined on further incubation. In a similar experiment in which the medium contained bovine serum albumin, it was noted that the decline of activity was postponed. In subsequent experiments in which the effects of other factors were tested, the adipose tissue was incubated in the experimental medium for a 40-minute period.

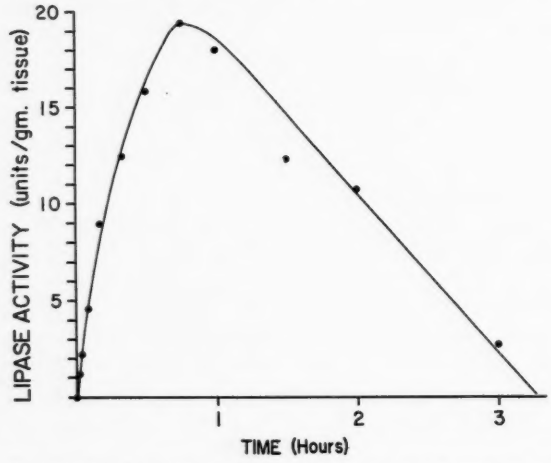

FIG. 1. The liberation of lipoprotein lipase by heparin from adipose tissue into Krebs-Ringer phosphate buffer per unit of time.

Demonstration of the Identity of Adipose Tissue Lipase. As a means of demonstrating that the lipolytic enzyme released into the medium by surviving adipose tissue was identical to lipoprotein lipase, the effects of some factors known to influence the activity of lipoprotein lipase were investigated. The results of these studies are presented in Table 1. Sodium chloride solution, 1 M, and protamine sulfate, which have been shown to be inhibitors of lipoprotein lipase, were also effective in inhibiting the activity of the enzyme released by adipose tissue. Sodium fluoride, on the other hand, which is known to inhibit pancreatic lipase but not lipoprotein lipase, had little or no effect. It will be observed that the inhibition produced by 1 M sodium chloride, while considerable, was not as complete as that reported by Korn (3) in experiments in which glycerol production was used as an index of enzyme activity. When free fatty acid production is measured, however, it has never been possible to show total inhibition of the activity of postheparin plasma by 1 M sodium chloride.[2]

The requirements of lipoprotein lipase for optimum activity on artificial fat emulsions were shown to apply also to the enzyme released from adipose tissue. Small amounts of fresh serum in addition to albumin markedly increased the lipolytic activity (see Table 2). In this experiment, of course, no fresh serum was added to the substrate mixture in the control incubation. The optimum pH for the activity of the lipase was shown to be in the range of 8.5 to 8.7, in good agreement with the figures reported for lipoprotein lipase by Korn and Quigley (6).

Finally, the ability of calcium phosphate gel to adsorb the enzyme was demonstrated. As may be seen in Table 3, the gel adsorbed the lipase completely, and a good recovery of a partially purified enzyme could be obtained by subsequent elution. This method of purifying lipoprotein lipase from postheparin plasma has been described by Nikkilä (11).

Conditions for the Release of Lipoprotein Lipase by Adipose Tissue. As noted above, there was no demonstrable release of lipoprotein lipase in the absence of added heparin. At heparin concentrations as low as 10 μg. per ml. some liberation of lipase could be observed. The optimal heparin concentration was found to be 30 μg. per ml.; at higher levels heparin itself has been found to inhibit the action of lipoprotein lipase (3), and to interfere with the subsequent enzyme assay.

Since many polyvalent anions have a heparinlike effect in eliciting the release of lipoprotein lipase into the circulation of the intact animal (8, 12, 13), an

[2] H. S. Gates and R. S. Gordon, Jr., unpublished data.

TABLE 1. EFFECTS OF INHIBITORS ON ADIPOSE TISSUE LIPASE

Inhibitor	Concentration	Percentage of Inhibition of Lipolysis
NaCl	1 M	83
NaF	0.2 M	7
Protamine sulfate	20 mg. per ml.	57

experiment was performed to test the effect of one such agent on the isolated adipose tissue. Sodium polymetaphosphate, in a concentration of 30 μg. per ml., was found to be effective, though less active than heparin, in stimulating the output of lipase from the tissue.

Experiments were also designed to test the effects of variations in the composition of the incubation medium on the liberation of lipase from adipose tissue, heparin being present in all cases at the optimal concentration of 30 μg. per ml. Differences in lipase liberation when albumin was added to the medium, when bicarbonate was substituted for phosphate, when simple isotonic saline (buffered with albumin) was substituted for the Krebs-Ringer solution, or when the incubation was carried out under anaerobic conditions, were not statistically significant at the 5 per cent level.

Comparison of Adipose with Other Tissues. The effectiveness of epididymal adipose tissue in releasing lipoprotein lipase into the incubation medium suggested that fat tissue might be chiefly responsible for the liberation of lipase into the plasma after injection of heparin into the intact animal. It therefore seemed desirable to compare the effectiveness of adipose tissue *in vitro* with that of other tissues from the same animal. A rat fed *ad libitum* was sacrificed, and the epididymal fat bodies, mesenteric fat, aorta, diaphragm, and slices of liver, kidney, spleen, and heart were incubated in Krebs-Ringer phosphate buffer containing 30 μg. of heparin per ml. The two samples of adipose tissue (epididymal and mesenteric) were most active in liberating lipase into the medium. The contrast was heightened when activity was expressed as units of lipase released per mg. tissue protein (estimated by biuret reaction). In subsequent experiments these differences were studied in greater detail with a larger number of rats.

Effect of Nutritional State of Donor Rats. In preliminary experiments, variations in the amount of lipoprotein lipase released under similar conditions suggested that differences in the nutritional state of the donor rat might be important in determining the amount of lipase released during incubation of the tissue with heparin *in vitro*. It had also been observed that the adipose tissue of fasted rats yielded less lipoprotein lipase than that of rats fed *ad libitum*, when the enzyme was prepared from extracts of acetone powders according to Korn (8). An experiment was therefore devised to emphasize the contrast between tissues from fasted and fed donor animals. Three rats were kept overnight with access to water but no food, and three others were given rat chow *ad libitum* and, in addition, had access to water containing 10 per cent sucrose. The epididymal fat bodies were removed from these rats and incubated in Krebs-Ringer phosphate buffer containing heparin at the usual concentration of 30 μg. per ml. In this study the contralateral tissues could not be used as controls, so that fasted and fed rats were compared. The differences observed were striking (Table 4): the tissues from the fed animals were much more active than those from fasted ones. Identical experiments were carried out to investigate the possibility that the nutritional state of the donor animal might influence the production of lipoprotein lipase by tissues other than epididymal fat. The results with mesenteric fat, diaphragm, and slices of myocardium and kidney are also summarized in Table 4. Only the samples of adipose tissue yielded differences that were statistically significant at the 5 per cent level.

TABLE 2. STIMULATION OF LIPOLYSIS BY ADDED SERUM
(0.1 ml. serum per ml. substrate)

Experiment	Lipase Activity Without Serum	Lipase Activity With Serum
1	4.8	36.5
2	5.1	36.4
3	2.5	18.2
4	2.3	23.3

DISCUSSION

The ability of adipose tissue to release lipoprotein lipase into the incubation medium on stimulation with heparin *in vitro* cannot be considered surprising in view of the demonstration by Korn and Quigley (5, 6) that this tissue is rich in enzyme activity, and the recent results of Robinson and Harris (14), which attest to the ease and rapidity of the release of lipoprotein lipase *in vivo*. The most noteworthy features

TABLE 3. Adsorption of Lipase by Calcium Phosphate Gel *

Fraction	Lipase	Protein	Specific Activity
	units/ml.	*mg./ml.*	
Original Incubation Medium	5.0	0.24	21
Supernatant from Gel Adsorption	0.1	0.17	0.6
Enzyme Eluted from Gel	3.3	0.026	127

* 5 ml. of tissue incubation medium were adsorbed with 0.5 ml. gel. The gel was washed with 0.1 M sodium oxalate, and the enzyme then eluted with 5 ml. 0.04 M sodium citrate.

of the present investigation are felt to be, first, the promptness of the liberation of lipase from adipose tissue by heparin and the apparent lack of any cofactor requirements and, second, the marked effect of the nutritional state of the donor animal in determining the activity and availability of lipase in the tissue.

It has been found in these studies that lipoprotein lipase is released into the medium when heparin or a heparin analogue (polymetaphosphate) contacts surviving adipose tissue *in vitro*. The composition of the medium and the availability of oxygen to support tissue respiration appear to be of no importance. In the light of the data of Korn and Quigley (5, 6), who have shown the existence of preformed enzyme in adipose tissue, it seems likely that the role of heparin is to attack the bond which normally causes the lipase to remain in the tissue, and thus make it free to dissolve into the medium. Heparin is not alone in possessing this ability, but it has been found (8, 12, 13) that many polyvalent anions may elicit the release of lipoprotein lipase *in vivo*. It may therefore be reasonable to suggest that lipoprotein lipase is normally held to cell surfaces primarily by electrostatic forces, perhaps acting between some cationic group on the cell surface and some polyvalent anionic grouping on the enzyme itself. The presence of small amounts of lipoprotein lipase in blood plasma under normal circumstances (15) is compatible with the hypothesis that the binding of the enzyme to cell surfaces is an equilibrium that normally allows slight dissociation.

The second important finding, and one which was unexpected, was the striking difference between tissues from fed and fasted animals with respect to the amount of lipoprotein lipase which could be liberated. As has been shown in previous studies (16), adipose tissue has a physiologic role in the release of stored fat into the blood stream as free fatty acid. It was anticipated that starvation, the nutritional state associated with maximum release of fatty acids, would be associated with maximal lipoprotein lipase release. The actual result was quite the contrary, and lipoprotein lipase activity was observed to be lower in fasted tissue, either when it was liberated into an incubation medium by the action of heparin, or when it was directly isolated from the tissue, without added heparin, by the preparation of an acetone powder.

It has long been suspected, but never rigidly proved, that lipoprotein lipase plays an important role in the normal processes of removal of chylomicron fat from the circulating plasma (16). One more indication that this hypothesis is correct may be obtained by comparing the present results with the data of Bragdon and Gordon (17), in which it is shown that the *in vivo* uptake of chylomicron fatty acids by adipose tissue is greater in the carbohydrate-fed than in the fasted rat. The liberation of free fatty acid from adipose tissue, which has been shown to be an important means for the mobilization of depot fat when it is required as a source of energy, might also be a function of the lipoprotein lipase contained in adipose tissue. This suggestion, however, is not consistent with the observations of this study, as the adipose tissues of fasted animals, which are most active in releasing free fatty acids (18), are just those that have the least demonstrable enzyme activity, whereas the adipose tissues of carbohydrate-fed animals, which do not release free fatty acids, are richest in lipoprotein lipase.

TABLE 4. Effect of Nutritional State on Release of Lipase by Rat Tissues

Tissue	Lipase Activity Released *	
	Fed Rats	Fasted Rats
	units/gm. wet tissue	*units/gm. wet tissue*
Epididymal Fat	40.1 ± 10.0	1.2 ± 0.49
Mesenteric Fat	21.4 ± 1.0	0.45 ± 0.39
Diaphragm	5.9 ± 0.73	4.1 ± 1.5
Heart Slices	10.0 ± 2.2	9.1 ± 1.6
Kidney Slices	4.1 ± 1.7	2.7 ± 0.57

* The values given are the means ± one standard deviation.

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The role of the sympathetic nervous system in the metabolism of free fatty acids*†

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SUMMARY

Norepinephrine and epinephrine increased circulating levels of free fatty acids in humans and dogs. The increase was sustained in dogs during infusion of norepinephrine, but transient during epinephrine infusion, which also increased plasma sugar concentration. Anxiety or discomfort in humans and decrease in depth of pentobarbital anesthesia in dogs were accompanied by increased free fatty acid concentration, whereas ganglionic blockade with hexamethonium in dogs resulted in a prompt and sustained fall. In intact and adrenalectomized dogs receiving constant infusions of palmitic acid-1-C^{14}, the decrease in free fatty acid concentration following hexamethonium was shown to result from a reduction in the rate at which they were added to plasma. Only a slight additional reduction was produced by insulin. The effects of norepinephrine and epinephrine on free fatty acid concentration were readily demonstrated during ganglionic blockade, whereas peripheral adrenergic blockade with dibenamine inhibited the response to these amines. The response of free fatty acids to sympathetic amines was also diminished in adrenalectomized dogs maintained with desoxycorticosterone alone. The concept is proposed that the sympathetic nervous system exerts a tonic action on the mobilization of fatty acids from adipose tissue which may be altered by central stimuli as well as by hormonal factors.

Recent studies (1) indicate that lipids are transported to peripheral tissues from adipose tissue depots mainly in the form of free fatty acids (FFA). These fatty acids are released from adipose tissue triglycerides and circulate in the blood plasma chiefly in a complex with albumin. The plasma level of FFA is increased by fasting and by administration of epinephrine (2, 3), growth hormone (4), and thyroid hormone (5). Epinephrine apparently acts by increasing the rate of mobilization of FFA. Partial glycerides accumulate in adipose tissue after administration of epinephrine (6), and *in vitro* studies have shown that epinephrine increases the rate of liberation of FFA from adipose tissue (7). Insulin, on the other hand, results in a fall in the plasma level of FFA (2, 3) and decreases the rate of FFA liberation from adipose tissue *in vitro* (7). It appears likely that a number of hormones can alter the rate of mobilization of FFA from adipose tissue. Results of earlier studies, however, have suggested that mobilization of fat from adipose tissue is influenced by the nervous system. Peripheral nerve endings supply not only the blood vessels of adipose tissue but also the fat cells themselves (8). A number of investigators have shown that interference with the innervation of adipose tissue may impair the ability of the depot to mobilize fat. Wertheimer (9) found that section of the spinal cord above the sixth thoracic segment prevented fatty liver in phlorhizinized dogs. According to Beznák and Hasch (10), Hausberger (11), and later Clément (12), unilateral denervation of various bodies of fat in the mouse, rat, cat, and rabbit diminishes the rate of depletion of triglycerides from the depot on the denervated side in fasting animals. In view of these findings, further characterization of the interrelationship between fat mobilization and the sympathetic nervous system seemed pertinent.

To assess the role of the sympathetic nervous system in the metabolism of plasma FFA, the effects of norepinephrine (the physiologic neurotransmitter re-

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‡ This work was performed during tenure as an Established Investigator of the American Heart Association.

leased at postganglionic sympathetic nerve endings [13]), ganglionic blockade, and peripheral adrenergic blockade were studied. These experiments indicate that the metabolism of FFA may be altered strikingly by changes in the activity of the sympathetic nervous system, as well as by administration of norepinephrine, and therefore furnish considerable support for the concept that the sympathetic nervous system may play an important role in the regulation of the mobilization of fat from adipose tissue.

METHODS

Experimental Subjects. Studies were performed on men and male dogs in the postabsorptive state. Human subjects were kept at rest in bed during study with indwelling needles in place in an antecubital vein. Most of the animals were anesthetized with pentobarbital; a few experiments were performed on unanesthetized animals to determine the effects of anesthesia per se. Dogs were adrenalectomized in two-stage operations and maintained on desoxycorticosterone (2.5 mg. daily, intramuscularly). In some instances cortisone (5 to 40 mg. daily, intramuscularly) was also given. In anesthetized dogs, indwelling catheters in the femoral artery were utilized for recording blood pressure and withdrawing blood samples. In unanesthetized dogs, blood samples were taken from an antecubital vein.

Agents Administered. All agents were injected intravascularly. Standard pharmaceutical preparations of epinephrine hydrochloride and norepinephrine bitartrate were used. Hexamethonium was administered as the bromide or chloride. Dibenamine was dissolved in equal parts of ethanol and propylene glycol to make a 5 per cent solution which was acidified with 0.5 N HCl 1:100. This solution was diluted with 0.15 M saline for injection. Palmitic acid-1-C^{14}, specific activity 5 mc. per mmole, was obtained from a commercial source. The acid was converted to the sodium salt and complexed with dog serum freed of lipoproteins less dense than 1.21, as described previously (14). The final molar ratio of fatty acid to albumin did not exceed 2.

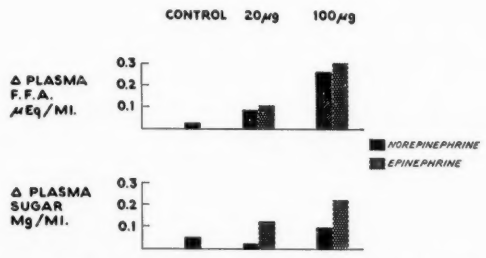

FIG. 1. Effect of graded doses of norepinephrine and epinephrine on plasma levels of FFA and sugar in a healthy 33-year-old male subject. The 20 μg. doses were administered in 2 minutes and the 100 μg. doses in 6 minutes. The increments plotted were those occurring 10 to 13 minutes after start of the infusions.

Analytical. Blood samples were mixed with 1 mg. per ml. of sodium oxalate, chilled immediately in ice water, and centrifuged at 3°C. The plasma was kept at −19°C until analyses were performed. No change in analytical values was observed in tests on plasma frozen for as long as 1 month. Packed cell volume was determined by centrifuging the blood samples at 1800 × *g* for 30 minutes in standard Wintrobe tubes, total protein by the method of Gornall *et al.* (15), and reducing substances by the method of Folin and Wu (16). FFA were extracted from plasma by the method of Davis (17). The ether extract was evaporated under an air stream at room temperature, and the lipids were taken up in heptane. FFA were titrated according to the method of Dole (2); radioactivity was determined in a Packard liquid scintillation spectrometer with 0.3 per cent diphenyloxazole in toluene as phosphor.

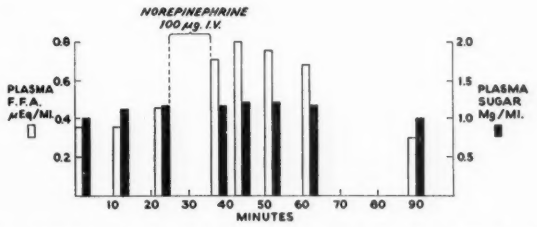

FIG. 2. Effect of norepinephrine on plasma levels of FFA and sugar in a healthy 35-year-old male subject.

RESULTS

Human Studies. The results of infusion of epinephrine and norepinephrine in a healthy subject are shown in Figure 1. The two agents had a similar effect on plasma FFA concentration, but epinephrine had a considerably more pronounced influence on plasma sugar than norepinephrine. In a second subject, as shown in Figure 2, infusion of 100 μg. of norepinephrine produced a rise in plasma FFA concentration which persisted for approximately 30 minutes. Absence of anxiety or discomfort in the subject was found to be essential for proper evaluation of the action of sympathetic amines on FFA. In three separate studies FFA concentrations rose 0.1 to 0.3 μeq. per ml. during a 30-minute "control" period following placement of indwelling needles.

Dog Studies. The depth of anesthesia was found to affect markedly the concentration of FFA in the plasma. In general, decrease of anesthetic effect to the point where the dog shivered or developed tachypnea was associated with a significant rise in FFA concentration. In four experiments plasma FFA levels rose 0.28, 0.30, 0.55, and 0.68 μeq. per ml., respectively, over a period of from 20 to 40 minutes as depth of anesthesia decreased. When anesthesia was properly maintained, single injections of small amounts of sympathetic amines produced detectable effects (Fig. 3).

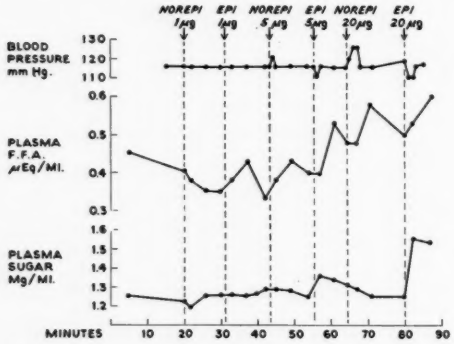

FIG. 3. Effect of graded doses of norepinephrine and epinephrine on plasma levels of FFA, sugar, and blood pressure in an anesthetized fasting male dog weighing 15 kg.

Prolonged infusion of norepinephrine (Fig. 4) resulted in persistent elevation of FFA concentration but no appreciable increase in sugar concentration, whereas similar infusion of epinephrine resulted in transient elevations of FFA and sustained elevation of sugar (Fig. 5). Packed cell volume rose significantly during these infusions, but plasma protein concentration was not significantly altered. The hemodynamic effects of

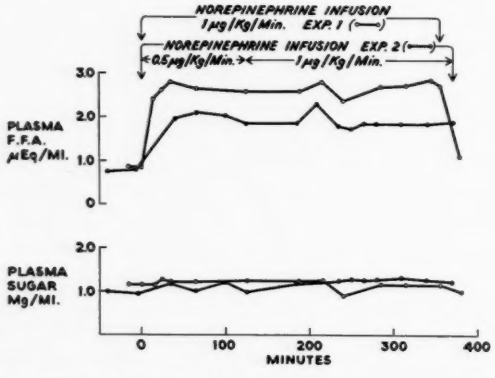

FIG. 4. Effect of norepinephrine infusion on plasma levels of FFA and sugar in 2 anesthetized fasting male dogs weighing approximately 20 kg.

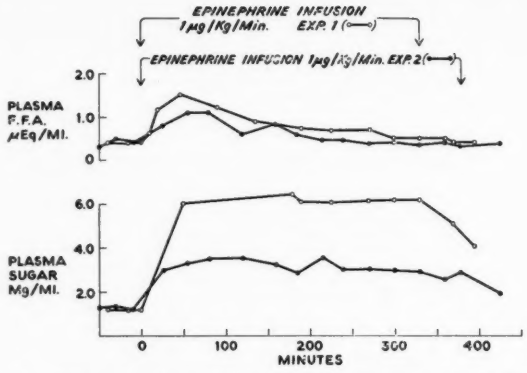

FIG. 5. Effect of epinephrine infusion on plasma levels of FFA and sugar in 2 anesthetized fasting male dogs weighing approximately 20 kg.

the infusions were transient; blood pressure remained elevated for only a few minutes.

Administration of hexamethonium uniformly resulted in a rapid and profound fall in plasma FFA concentration whenever the initial level was normal or high. The decrease persisted for long periods when ganglionic blockade was maintained (Fig. 6). Infusion

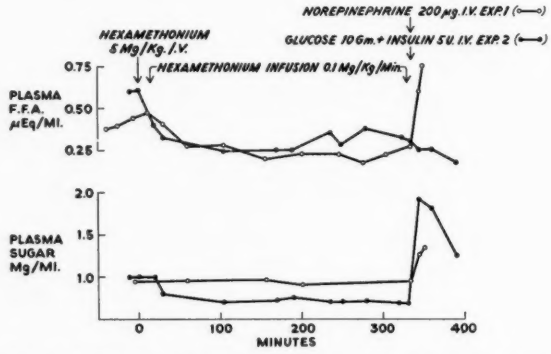
NOREPINEPHRINE 200 μg. I.V. EXP. 1
HEXAMETHONIUM 5 Mg/Kg. I.V.
GLUCOSE 10 Gm. + INSULIN 5 U. I.V. EXP. 2
HEXAMETHONIUM INFUSION 0.1 Mg/Kg/Min.
PLASMA F.F.A. μEq/Ml.
PLASMA SUGAR Mg/Ml.
MINUTES

FIG. 6. Effect of hexamethonium administration on plasma levels of FFA and sugar in 2 fasting male dogs weighing approximately 20 kg. Pentobarbital anesthesia. Additional doses of 1 mg. per kg. of body weight were given at intervals during experiment 1 to maintain ganglionic blockade. The subsequent effects of glucose and insulin in one experiment and norepinephrine in another are also shown.

of glucose and insulin after administration of hexamethonium produced only a slight additional fall in FFA concentration, but the effects of norepinephrine and epinephrine were readily demonstrated. The action of hexamethonium was not dependent on anesthesia since similar results were obtained in unanesthetized dogs. In four anesthetized dogs given hexamethonium decreases in depth of anesthesia to the point where they shivered produced no significant change in plasma FFA levels (<0.05 μeq. per ml.).

To determine whether the effects of hexamethonium resulted from alterations in the rate at which FFA was added to plasma or removed from it, the following studies were performed. A solution of palmitic acid-1-C^{14}, complexed with serum freed of lipoproteins less dense than 1.21, was infused into anesthetized fasting dogs at the constant rate of 1.4×10^5 cpm. per minute (0.15 μeq. FFA per minute). After an appropriate control interval hexamethonium was given intravenously. After another interval a second agent was administered. Serial samples of blood plasma were extracted and assayed for radioactivity and FFA. Fractionation of the extract (14) showed that almost all the radioactivity was present in FFA. The results of one such experiment are shown in Figure 7. The level of radioactivity was stable during the

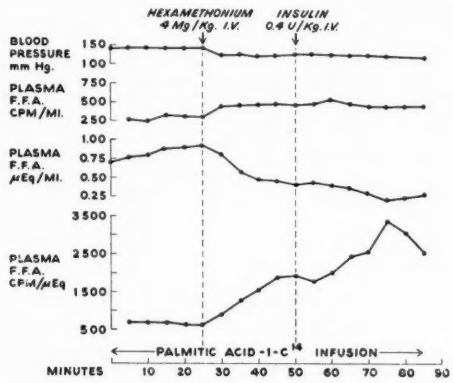

FIG. 7. Effect of hexamethonium and subsequent administration of insulin on blood pressure, plasma level of FFA, radioactivity in lipid extract of plasma, and calculated specific activity of FFA during constant infusion of palmitic acid-1-C^{14} in an anesthetized fasting male dog weighing 20 kg.

control period. After hexamethonium, total radioactivity rose slightly, indicating a decrease in the rate of removal of FFA from plasma. In spite of this, FFA concentration fell rapidly, showing that the rate of addition of FFA to plasma was greatly reduced. Administration of insulin was followed by a further slight fall in FFA concentration and a rise in FFA specific activity, suggesting an even greater decrease in the rate of addition of FFA to plasma. Additional studies showed that hexamethonium had little effect on plasma levels of FFA in dogs pretreated with glucose and insulin. Similar experiments were performed on two adrenalectomized dogs on a maintenance regimen of 2.5 mg. of desoxycorticosterone acetate and 40 mg. of cortisone daily. In the first dog (Fig. 8) FFA concentration was low initially, and only a slight fall was observed after hexamethonium. At the same time total radioactivity rose, so that FFA specific activity was

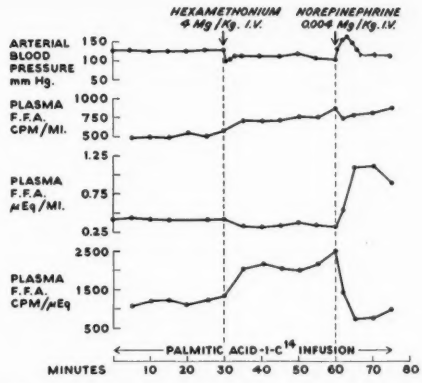

FIG. 8. Effect of hexamethonium and subsequent administration of norepinephrine on blood pressure, plasma level of FFA, radioactivity in lipid extract of plasma, and calculated specific activity of FFA during constant infusion of palmitic acid-1-C^{14} in an anesthetized fasting adrenalectomized male dog weighing 22 kg.

doubled, indicating a sharp reduction in the rate at which FFA was added to plasma. Administration of norepinephrine resulted in a striking increase in the rate at which FFA was added to plasma, as shown by a rise in FFA concentration with no change in total radioactivity. In the second adrenalectomized dog (Fig. 9) a decrease in anesthetic effect during the con-

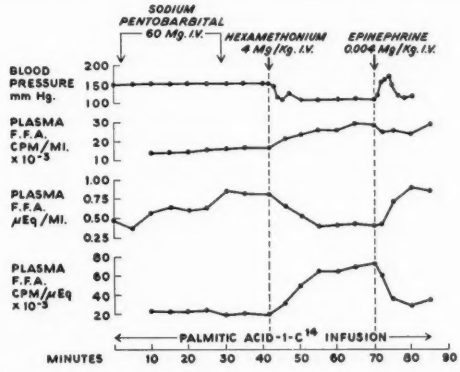

FIG. 9. Effect of hexamethonium and of subsequent administration of epinephrine on blood pressure, plasma level of FFA, radioactivity in lipid extract of plasma, and calculated specific activity of FFA during constant infusion of palmitic acid-1-C^{14} in an anesthetized fasting adrenalectomized male dog weighing 18 kg. Additional doses of pentobarbital were required to maintain anesthesia during the control period.

trol period was associated with a significant rise in FFA concentration. The effects of hexamethonium and epinephrine were similar to those noted in the previous experiment.

In contrast to their striking action in adrenalectomized animals maintained with cortisone in addition to desoxycorticosterone, epinephrine and norepinephrine had only a relatively slight effect on adre-

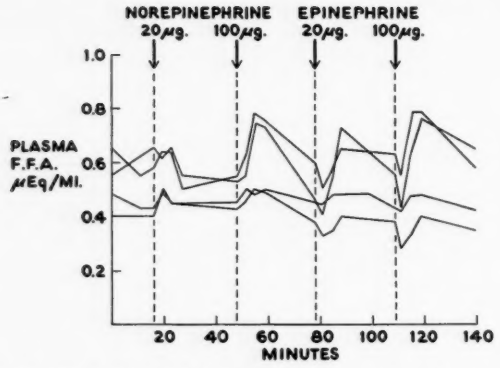

FIG. 10. Effect of graded doses of norepinephrine and epinephrine on plasma level of FFA in anesthetized adrenalectomized male dogs weighing 17 to 23 kg. Therapy: 2.5 mg. desoxycorticosterone acetate intramuscularly daily for 6 to 12 weeks prior to study.

nalectomized dogs maintained with desoxycorticosterone alone. Only two of the four dogs studied showed a significant rise in plasma FFA concentration after administration of either agent in doses up to 100 μg. (Fig. 10). Administration of cortisone (10 mg. daily) restored the sensitivity to catechol amines in a dog that had shown no response when receiving desoxycorticosterone alone. The level of FFA increased 0.33 and 0.25 μeq. per ml. of plasma 10 minutes after administration of 100 μg. of norepinephrine and epinephrine respectively. The transient fall in FFA concentration after epinephrine (Fig. 10) was a consistent finding; it took place at the time the hemodynamic effects of epinephrine were subsiding. No such fall was observed after administration of norepinephrine.

The effects of norepinephrine and epinephrine on plasma FFA were blocked by prior intravenous administration of dibenamine (20 mg. per kg. of body weight). Because dibenamine was found to produce a prompt rise in plasma FFA and sugar concentration, several hours were allowed to elapse before experiments were carried out on the treated animals. The results of one such experiment in an unanesthetized dog, given in Figure 11, showed that dibenamine had blocked the effect of sympathetic amines on FFA, whereas the effect of epinephrine on plasma sugar was unaltered. Two other experiments in anesthetized dogs gave similar results, and blood pressure measurements showed reversal by dibenamine of the usual effect of the sympathetic amines.

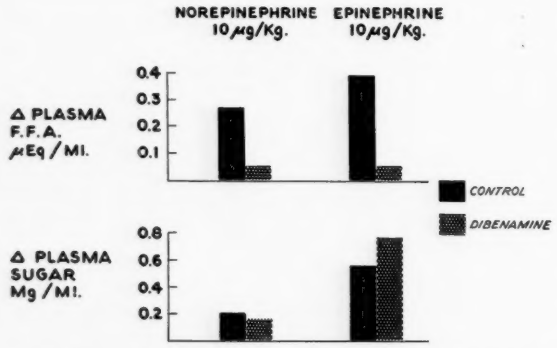

FIG. 11. Effect of administration of dibenamine on response of plasma levels of FFA and sugar to large doses of norepinephrine and epinephrine. Anesthetized fasting male dog weighing 16 kg. Control experiment performed 3 weeks prior to administration of dibenamine. The changes plotted were those occurring 10 minutes after administration of the amines.

DISCUSSION

The observation that norepinephrine, like epinephrine, results in a rapid increase in the concentration of plasma FFA indicates that norepinephrine has important metabolic functions. Brewster *et al.* (18), in a study on anesthetized dogs under total epidural sympathetic blockade, found that infusion of epinephrine and norepinephrine led to an equal rise in oxygen consumption, but that only epinephrine produced a rise in serum lactate, pyruvate, and sugar concentration. We also observed an increase in oxygen consumption associated with an increase in plasma FFA concentration in anesthetized dogs after administration of norepinephrine.[1] The possible importance of the effect of norepinephrine on plasma FFA is suggested by the profound decrease in the rate at which FFA is added to plasma after administration of hexamethonium. This change, presumably reflecting decreased mobilization of adipose tissue triglycerides, is most likely the result of decreased liberation of norepinephrine from sympathetic nerve endings in the parenchyma of adipose tissue. Blockade of adrenal medullary secretion cannot be the decisive alteration, since the response to hexamethonium was similar in intact and adrenalectomized dogs. The perfusion of adipose tissue after ganglionic blockade has not been studied. Therefore the role of changes in blood flow in adipose tissue cannot be assessed in these experiments. Regardless of the mechanism, however, the action of hexamethonium on FFA mobilization must be assumed to result from prevention of the release of norepinephrine, since the action of injected norepinephrine is unaltered in the animal under ganglionic blockade.

Our interpretation of the experiments in which palmitic acid-1-C^{14} was infused requires the assumption that palmitic acid is a representative tracer for FFA

[1] Unpublished observations.

as a whole. In man the bulk of plasma FFA consists of palmitic, oleic, and stearic acids (19), the first two of which have a similar turnover rate (20). If these results can be extended to dogs, this assumption would be reasonable.

The rise in plasma FFA accompanying anxiety or discomfort in humans and decreasing depth of anesthesia in dogs provides further evidence of the role of the sympathetic nervous system in the mobilization of fatty acids from adipose tissue. Pentobarbital anesthesia in dogs is accompanied by diminished adrenal secretion of norepinephrine and epinephrine. Decreases in the depth of anesthesia are associated with increases in medullary secretion.[2] This effect of anesthesia may apply to the extra-adrenal sympathetic nervous system as well, since lessening of anesthesia in an adrenalectomized dog was accompanied by a rise in plasma FFA concentration (Fig. 9). Furthermore, alterations in depth of anesthesia did not result in changes in plasma FFA concentration in dogs given hexamethonium.

Administration of hexamethonium to dogs receiving a constant infusion of palmitic acid-1-C^{14} resulted in an increase in plasma radioactivity, indicating a decrease in the rate of removal of FFA from plasma. This decrease might occur as the result of reduced blood flow. In addition, the transient fall in FFA concentration after injection of epinephrine may well result from the increased cardiac output produced by this agent, particularly since norepinephrine, which presumably does not alter cardiac output appreciably, did not produce a similar reduction in FFA. Considering the extremely rapid turnover of FFA in the blood (14), it is not surprising that the rate of blood flow may limit the rate of their removal from the circulation.

In our experiments administration of dibenamine blocked the response of FFA to norepinephrine and epinephrine, but failed to lower FFA concentration to levels observed after administration of hexamethonium. This observation is in keeping with previous ones that this agent blocks the hemodynamic effects of injected sympathetic amines more effectively than the effects of spontaneous sympathetic activity (21). Since dibenamine must also block the effects of epinephrine and norepinephrine liberated from the adrenal medulla, it follows that adrenal medullary secretion was not an important factor in maintenance of plasma FFA levels in the dogs studied. Furthermore, we have observed that adrenalectomized dogs maintained with desoxycorticosterone acetate and cortisone have normal plasma FFA levels.[3] The failure of dibenamine to block the effects of epinephrine on plasma sugar has been reported previously (22). This dissociation, in conjunction with the fact that epinephrine and norepinephrine have similar effects on FFA but quite different effects on sugar concentration, suggests that these agents utilize different cellular mechanisms to effect release of FFA from adipose tissue and to promote glycogenolysis in the liver. The rise in plasma FFA and sugar concentration during the period of development of dibenamine blockade is consistent with other evidence which suggests that adrenal medullary discharge occurs during this time (23).

The response of the plasma FFA of adrenalectomized dogs to administration of sympathetic amines is consistent with the concept that the effects of sympathetic amines are decreased in the absence of adrenal cortical function (24). Shafrir *et al.* (25) obtained somewhat similar results with epinephrine.

The importance of extra-adrenal sympathetic activity in fat mobilization is supported by the recent demonstration by White and Engel (26) that L-norepinephrine is approximately as effective as L-epinephrine in promoting release of FFA from rat epididymal adipose tissue incubated *in vitro*, and the report of Levy and Ramey (27) that administration of ergotamine tartrate prevented loss of lipid from epididymal adipose tissue in fasting adrenalectomized rats.

The physiologic significance of the effect of sympathetic nervous activity on the mobilization of fatty acids from adipose tissue is not defined precisely by this study. It is clear, however, that this mechanism can provide for rapid mobilization of fatty acids for energy needs of peripheral tissues. The reduction in plasma FFA levels in fasting dogs produced by hexamethonium suggests that the tonic activity of the sympathetic nervous system may provide for continuous release of FFA at a level which can be modified by insulin and other humoral agents. These observations indicate the need for further investigation of the interplay of nervous and humoral factors in the homeostatic control of fatty acid mobilization in various physiologic and pathologic states, and of the secondary metabolic effects in liver and peripheral tissues of altered mobilization of FFA.

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Volume 1
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The nature of the epinephrine-induced hyperlipidemia in dogs and its modification by glucose*

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SUMMARY

Dogs receiving subcutaneous epinephrine in oil (1 mg. per kg.) showed a prompt but transient elevation in serum free fatty acids (FFA) and a delayed 24-hour elevation of serum lipoproteins. On daily injections of epinephrine for 6 to 8 days the serum cholesterol rose to 91 per cent above control values and the phospholipid 53 per cent. The triglyceride response was smaller and quite variable. Little change was found in the $d < 1.019$ lipoprotein, a three to eightfold increase in the $d = 1.019$ to 1.063 fraction and a 15 to 41 per cent increase in the $d = 1.063$ to 1.21 fraction. Epinephrine prolonged alimentary lipidemia but did not inhibit disappearance of intravenously infused chylomicrons. By prior and concomitant administration of glucose the FFA elevation after epinephrine was prevented. Insulin alone also blocked the epinephrine-induced FFA response without parallel hyperglycemia, indicating that availability of glucose for effective tissue utilization rather than hyperglycemia per se controls the release of FFA. Despite the block in the FFA response to epinephrine by either glucose or insulin, there was a definite elevation of serum cholesterol and phospholipids at 24 hours, suggesting an at least partially independent lipoprotein mobilizing action of the hormone. The relation of these findings to stress-induced hypercholesterolemia is considered.

In 1928 Cori and Cori (1) showed that epinephrine-induced hypermetabolism was accompanied by a depression of the respiratory quotient, implying an increased utilization of fat. The nature and the mechanism of the lipid mobilization due to epinephrine, however, deserves re-examination in the light of recent advances in the field of fat metabolism.

In early investigations with aqueous epinephrine solutions, diverse or inconsistent changes in blood lipid levels were noted (2). More recently it has been reported that administration of long-acting epinephrine in oil causes a rise in serum cholesterol, phospholipids, and triglycerides of dogs and rabbits (3, 4). Epinephrine in aqueous solution has also been demonstrated to raise considerably the blood level of free fatty acids (FFA) in humans (5, 6) as well as to mobilize FFA from rat adipose tissue *in vitro* (7).

This investigation was carried out to determine the nature of the epinephrine-induced hyperlipidemia and to establish whether any relationship exists between the increases in circulating FFA and the elevation of other blood lipids following epinephrine administration.

METHODS AND EXPERIMENTAL PROCEDURES

Experiments were performed on mongrel dogs weighing 9 to 17 kg. They were maintained on a dry meal of 7 per cent fat content offered each morning for 4 hours. In the long-term studies six dogs received a single dose of 1 mg. per kg. epinephrine in peanut oil,[1]

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[1] Supplied by Dr. J. E. Gajewski of Parke, Davis and Co.

administered subcutaneously, daily at 9 A.M. for a period of 6 to 8 days. Venous blood samples were drawn after a 20-hour fast, just before the daily dose of epinephrine was given. The blood was promptly chilled and kept at 4°C until the analytical procedures were started, within 2 hours. In six fasting dogs changes in serum lipid constituents were measured during the 24 hours following a single injection of epinephrine in oil. For the sake of comparison, an equivalent amount of epinephrine in saline solution (Adrenalin Chloride®) was infused over a period of 5 hours to two fasting dogs anesthetized with Pentothal®.

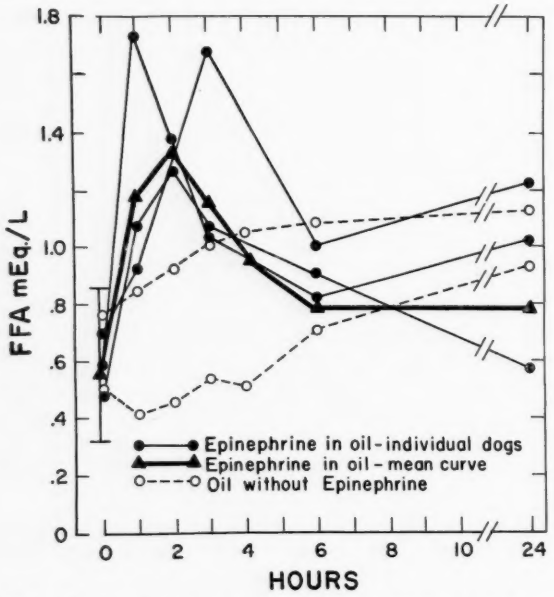

FIG. 1. Serum FFA levels during 24 hours following a single injection of epinephrine in oil. The vertical bar parallel to the ordinate indicates the range of normal fasting FFA values in 24 dogs used throughout this study.

In the fat load experiments, whipping cream was introduced by stomach tube or a specified amount of canned meat [2] was given to six fasting dogs and the serum lipid levels were determined at intervals over the following 24 hours. A week later the same animals received the fat load 30 minutes after a single subcutaneous dose of epinephrine in oil (1 mg. per kg.).

The rate of disappearance of intravenously injected chylomicrons was measured in untreated dogs and in the same dogs 30 minutes after subcutaneous injection of epinephrine in oil (1 mg. per kg.). Chylomicron disappearance was measured by a method similar to that described by Havel and Fredrickson (8). Thoracic duct chyle obtained from cream-fed donor dogs served as the source of chylomicrons. Chyle was injected into the external jugular vein of the recipient dogs under Pentothal® anesthesia in an amount representing 100 mg. triglyceride per kg. body weight. Blood samples were withdrawn at timed intervals from the femoral artery. The samples were delivered into heparinized tubes, kept on ice for 1 hour and centrifuged at approximately $1800 \times g$. The optical density of the supernatant whole plasma was read in a Coleman Junior spectrophotometer at a wave length of 660 mμ. It has been shown that the curves obtained by this simple procedure parallel those obtained when the chylomicrons are first isolated ultracentrifugally and then suspended in 0.9 per cent sodium chloride solution for the measurement of optical density or when chylomicron triglyceride content is determined.[3]

In the study of the effect of carbohydrate on epinephrine-induced 24-hour serum lipid changes, 30 g. of glucose in a 30 per cent aqueous solution was administered by stomach tube, followed by five doses of 15 g. each, at 30-minute intervals. Immediately after the second dose, the dog received a subcutaneous injection of epinephrine in oil (1 mg. per kg.). Each animal served as its own control in an experiment carried out in the same manner with water substituted for the glucose solutions.

The effect of insulin alone on the epinephrine-induced hyperlipidemia was explored by injecting subcutaneously a mixure of from 2 to 3 units per kg. of crystalline insulin and from 1 to 1.5 units per kg. of long-acting insulin (NPH or Protamine Zinc insulin) at the same time as epinephrine in oil. In five out of eight experiments 50 g. of glucose was given by stomach tube 6 hours after the insulin injection, to prevent the development of hypoglycemia due to the long-acting insulin preparation. Each animal served as its own control, receiving the same dose of epinephrine without insulin.

Serum free fatty acids (FFA) were determined by a modification of the method of Gordon (9). Total lipids and triglycerides were determined by the method of Bragdon (10). Cholesterol was measured by the Sperry-Webb procedure (11), and lipid phosphorus by a modification of the procedure of Stewart and Hendry (12). Blood glucose was measured colorimetrically by the Somogyi-Nelson method (13).

Ultracentrifugal separation of serum lipoprotein fractions was performed according to the procedure of Havel *et al.* (14).

[2] Red Heart Dog Food, John Morrel and Co., containing 5.2 per cent total fat, as estimated from dichromate oxidation value (10).

[3] Dr. D. M. McCollester, personal communication.

TABLE 1. SERUM LIPID LEVELS (MG./100 ML.) DURING 24 HOURS FOLLOWING A SINGLE INJECTION OF LONG-ACTING EPINEPHRINE

Hours After Injection	Total Cholesterol				Free Cholesterol				Phospholipids				Triglycerides			
	Dog No. 1	Dog No. 4	Dog No. 6	Control	Dog No. 1	Dog No. 4	Dog No. 6	Control	Dog No. 1	Dog No. 4	Dog No. 6	Control	Dog No. 1	Dog No. 4	Dog No. 6	Control
0	143	125	117	130	38	37	33	32	342	299	266	296	29	75	42	31
3	149	123	115	126	36	36	32	33	370	329	258	283	68	29	35	44
6	151	134	129	132	41	39	36	33	360	299	291	284	45	65	47	30
24	165	173	155	133	46	60	47	35	418	444	384	316	70	67	48	55

RESULTS

Serum Lipid Changes in Dogs After a Single Dose of Epinephrine. Figure 1 presents the changes in FFA concentration during the first 24 hours following the injection of epinephrine in oil in three out of nine animals studied, and the mean curve. The three representative curves demonstrate the prompt increase in serum FFA which, however, did not persist throughout the period of observation. The FFA levels reached a peak of from two to three times the initial value within 1 to 3 hours, returning toward the normal range by 6 hours. In the control dogs injected with peanut oil, the gradual increase in serum FFA concentration during the experimental period may be attributed to continuous fasting (5). The somewhat higher levels at 24 hours in both control and experimental animals may be similarly explained.

In contrast to the response of serum FFA, little or no significant increase in the concentration of other lipids was observed at 6 hours. However, 24 hours after the injection highly significant increases in the levels of cholesterol and phospholipids had occurred (Tables 1 and 2). The changes in triglyceride levels were smaller and highly variable as is indicated by the large standard deviation.

In order to establish whether the response to epinephrine might be affected by the manner of administration of the hormone, a saline solution of epinephrine was infused into a fasting, anesthetized dog. As shown in Figure 2, it was not possible, even by constant intravenous infusion of increasing amounts of epinephrine, to sustain the initial increase in serum FFA. The response of the other lipids was similar to that obtained with the long-lasting epinephrine preparation.

Effect of Long-term Administration of Epinephrine in Oil. The effect on serum lipids of daily admin-

TABLE 2. SERUM LIPID CHANGES IN DOGS TREATED DAILY WITH EPINEPHRINE *

No. of Days of Epinephrine Treatment	No. of Animals	Per Cent Increase ± SD of the Initial Lipid Level				FC/TC	TC/PL
		TC	FC	PL	TG		
						mg./mg.	*mg./mg.*
0	19					.279	.435
1	19	28.6 ± 7.9	49.6 ± 18.7	41.0 ± 13.9	24.8 ± 34.4	.323 †	.397 †
3	7	60.1 ± 25.8	79.5 ± 34.6	48.2 ± 23.6	33.2 ± 13.3	.301 ‡	.489 ‡
6-8	6	90.9 ± 50.0	93.5 ± 60.2	52.7 ± 22.5	16.9 ± 49.0 §	.273 §	.549 ‡

TC = total cholesterol FC = free cholesterol PL = phospholipids TG = triglycerides

* All serum lipid elevations are significant relative to the zero time control levels with a p value less than 0.01, except for the triglyceride level at 6-8 days, which is not significantly different from that at zero time. The FC/TC and TC/PL ratios represent mean values averaged over all sera collected at the indicated times. In both sets of data significance was evaluated on paired data, each dog serving as his own control.

† Significantly different from zero time value at $p < 0.001$.

‡ Significantly different from zero time value at $p < 0.05$.

§ Not significantly different from zero time value.

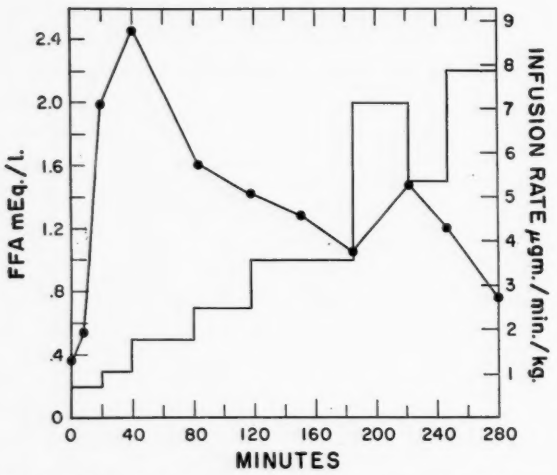

FIG. 2. Effect of continuous infusion of aqueous epinephrine on serum FFA levels. Twelve mg. of epinephrine in saline solution was infused into a Pentothal® anesthetized dog weighing 14 kg. The increasing rate of infusion is indicated by the steps in the figure.

istration of epinephrine in oil is represented in Figure 3 and Table 2.

The cholesterol and phospholipids, which became significantly elevated within 24 hours, remained at a high level as long as the animals continued to receive the hormone. It is of interest that the percentage rise of the free cholesterol fraction at 24 hours was considerably larger than that of esterified cholesterol, as is evidenced by the increased free to total cholesterol ratios. With continuation of epinephrine treatment, these ratios returned to normal.

The phospholipid elevation after one day of epinephrine treatment was proportionately greater than the rise in cholesterol resulting in lowered cholesterol to phospholipid ratios. The cholesterol level, however, continued to rise sharply, and after 6 to 8 days of treatment the cholesterol to phospholipid ratios became markedly increased when compared with the initial values.

The triglyceride levels have shown considerably more variability than those of the other serum lipids. This was true of the fasting control levels in various animals and also of the levels in the same animal at different times (Table 1). Furthermore, the triglyceride levels in treated animals fluctuated widely in contrast to the more sustained responses of the other lipids (Fig. 3). Whereas the cholesterol and phospholipid levels continued to rise with extended epinephrine treatment, the triglycerides did not. After 6 to 8 days they did not differ significantly from the zero time value.

When assessing the significance of the triglyceride changes, it should be stressed that in Tables 1 and 2 the changes are expressed as percentages of a component which is present in dog serum at low levels, and is determined by a method that depends upon calculation by difference (10).

Composition of Serum Lipoprotein Fractions in Dogs Maintained on Epinephrine. Results of ultracentrifugal separation of three main classes of lipoproteins from three dog sera are summarized in Table 3. The sera were obtained before and after 1 or 8 days of daily administration of epinephrine. The elevation in serum lipids caused by epinephrine was accounted for by rises in the d = 1.019 to 1.063 and 1.063 to 1.21 lipoproteins, the d < 1.019 fraction showing very little change.

The major change occurred in the d = 1.019 to 1.063 fraction, normally a very small component, where the percentage rise in total lipid content after 8 days of epinephrine treatment was 352, 700, and 220 per cent in the three animals studied. The percentage rise in the d = 1.063 to 1.21 fraction was considerably smaller (18, 15, and 41 per cent), but since this fraction is so much larger, the absolute increments, as shown in Table 3, nevertheless accounted for 39, 17, and 64 per cent of the total recovered lipid increment.

The elevation of the cholesterol to phospholipid ratio after 8 days of epinephrine treatment (Table 2) correlates with the marked elevation of the d = 1.019 to 1.063 fraction, in which this ratio is higher than it is in

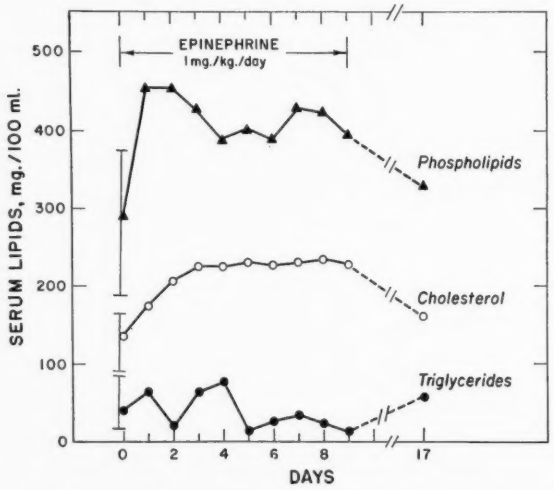

FIG. 3. Serum lipid response to prolonged administration of epinephrine in oil. Representative experiment in which a male dog weighing 14 kg. received subcutaneous injections of epinephrine in oil 1 mg./kg./day for 9 days. The vertical bars parallel to the ordinate indicate the range of normal fasting serum lipid values found in 19 dogs used in this study.

TABLE 3. COMPOSITION OF SERUM LIPOPROTEIN FRACTIONS IN DOGS TREATED WITH EPINEPHRINE

	mg./ 100 ml. of:	Dog No. 1			Dog No. 2			Dog No. 3				
		Control	Treated 8 Days	Net Change	Control	Treated 8 Days	Net Change	Control	Treated 1 Day	Net Change	Treated 8 Days	Net Change
Whole Serum	TL	633	910	*277*	546	995	*449*	547	820	*273*	811	*264*
	TC	155	231	*76*	126	277	*151*	130	176	*46*	214	*84*
	PL	364	518	*154*	296	487	*191*	283	481	*198*	449	*166*
	TG	41	54	*13*	65	98	*33*	69	83	*14*	43	*−26*
d < 1.019	TL	36	34	*−2*	63	59	*−4*	36	63	*27*	30	*−6*
	TC	4	4	*0*	3	8	*5*	5	7	*2*	4	*−1*
	PL	6	7	*1*	6	15	*9*	6	11	*5*	7	*1*
	TG	25	22	*−3*	53	34	*−19*	23	45	*22*	17	*−6*
d = 1.019-1.063	TL	35	193	*158*	50	400	*350*	45	101	*56*	144	*99*
	TC	8	53	*45*	14	114	*100*	11	27	*17*	43	*32*
	PL	16	85	*69*	23	180	*157*	15	46	*31*	57	*42*
	TG	10	35	*25*	9	65	*56*	14	15	*1*	18	*3*
d = 1.063-1.21	TL	567	669	*102*	426	491	*65*	422	592	*170*	595	*173*
	TC	144	165	*21*	103	128	*25*	104	133	*29*	157	*53*
	PL	341	406	*65*	260	292	*32*	236	377	*141*	345	*109*
	TG	10	15	*5*	10	4	*−6*	16	15	*−1*	18	*2*

TL = total lipids TC = total cholesterol PL = phospholipids TG = triglycerides

either of the other density classes. In addition, however, it is noteworthy that the lipid pattern within the density classes changed during epinephrine treatment. In both the d = 1.019 to 1.063 and the d = 1.063 to 1.21 classes the cholesterol to phospholipid ratio fell after 1 day of treatment and then returned to normal values after 8 days of treatment. These changes correlate with the significant drop in the ratio at 1 day recorded in Table 2.

Influence of Epinephrine on Serum Triglyceride Levels in Relation to Exogenous Fat Intake. It was necessary to evaluate any possible role of fat intake upon the hyperlipidemia induced by epinephrine, especially since epinephrine is known to decrease the motility of the gastrointestinal tract and possibly delay the processes of food absorption.

A single-dose high-fat meal was given to dogs and the serum lipid changes followed through 24 hours. The response of FFA, cholesterol, and phospholipids was similar to that in dogs fed a fat-poor diet. The duration of alimentary lipemia, however, was considerably prolonged by epinephrine (Fig. 4).

The Effect of Epinephrine on the Removal Rate of Blood Chylomicrons. The prolonged increase in triglyceride levels following epinephrine injection in the dog consuming large quantities of fat (Fig. 4) suggested that this might represent a retardation by epinephrine, either of the absorption of fat or of the removal of absorbed fat from the blood stream. Measurements of the disappearance time of intravenously injected chylomicrons were therefore undertaken. The curves shown in Figure 5 do not indicate any significant effect of epinephrine upon the half life of the circulating chylomicrons. It should be noted that the experimental methods here limit the accuracy of the determination of so short a half life. However, since the experiments were carried out in dogs serving as their own controls, each time being injected with comparable amounts of chylomicrons, and since the half-life values obtained were in no case prolonged by epinephrine, the conclusion that there is no significant retardation of chylomicron removal by epinephrine seems to be warranted.

Effect of Glucose Administration on the Epinephrine-induced Hyperlipidemia. It has been demonstrated previously that following a carbohydrate meal, plasma FFA concentrations are diminished and that the rate of release of FFA from adipose tissue *in vitro* is diminished by glucose (5, 6, 7). Experiments were performed to determine whether glucose given with epinephrine would inhibit the characteristic changes in FFA levels and whether any parallel

TABLE 4. EFFECT OF ADMINISTRATION OF GLUCOSE AND OF INSULIN ON THE EPINEPHRINE-INDUCED CHANGES IN SERUM LIPID LEVELS

	No. of Animals	Per Cent Elevation of Lipids *			
		TC	FC	PL	TG
Epinephrine only	3	30.7	44.5	43.0	12.5
Epinephrine + glucose		23.5	36.0	25.2	31.1
Epinephrine only	5	25.2	41.2	37.8	3.1
Epinephrine + insulin		16.8	28.5	23.0	2.9
Significance of difference between epinephrine and epinephrine + insulin-treated animals (paired data)		$p < 0.02$	$p < 0.01$	$p < 0.10$	not significant

TC = total cholesterol FC = free cholesterol PL = phospholipids TG = triglycerides

* Mean elevation measured 24 hours after injection of epinephrine in oil (1 mg/kg.).

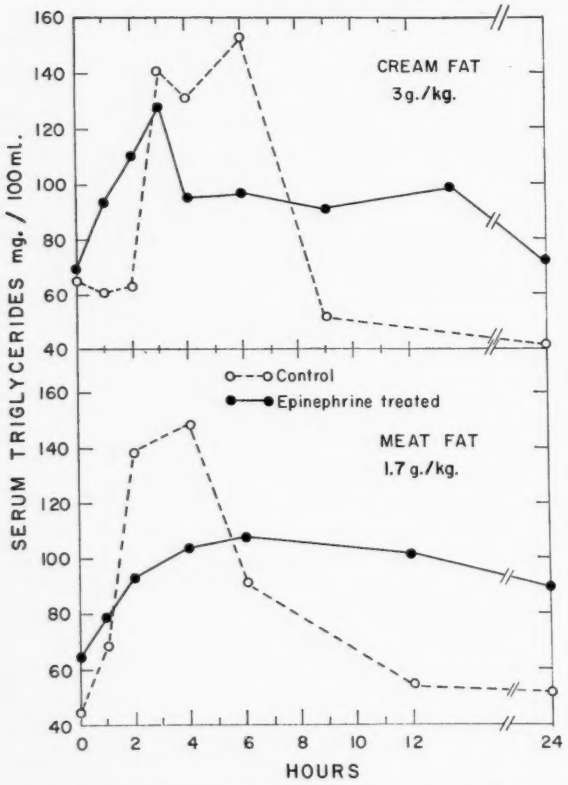

FIG. 4. Serum triglyceride levels in dogs after fat load. Representative experiments in 2 dogs receiving oral cream or meat fat loads with and without epinephrine in oil (1 mg./kg.).

effect would be observed on the epinephrine-induced changes in other serum lipids. Dogs receiving glucose by stomach tube were given a single injection of epinephrine in oil. It is seen from Figure 6 that in the control experiments in which epinephrine only was given, the peak FFA elevation occurred before the maximal epinephrine-induced hyperglycemia was reached. As the blood glucose rose, the FFA values returned toward normal. When glucose was administered to the same animal along with the epinephrine, the blood level of FFA dropped and then did not rise above normal (five experiments). On the other hand, as recorded in Table 4, feeding with glucose did not prevent the characteristic rise in cholesterol and phospholipids 24 hours after epinephrine, although there was some decrease in the magnitude of the response.

Effect of Insulin on Epinephrine-induced Hyperlipidemia. The demonstrated effect of glucose on the epinephrine-induced lipid mobilization prompted further attempts to modify the epinephrine response with insulin alone. It was of particular interest to explore whether the early elevation of blood glucose levels produced by glucose administration was essential for the effect on FFA described above.

As shown in Figure 7, insulin abolished the characteristic FFA response following epinephrine (eight experiments). At the same time the epinephrine-induced hyperglycemia was either completely prevented (five experiments) or sharply reduced (three experiments).

These results demonstrate that hyperglycemia per se is not essential to prevent the rise in FFA; the effects of glucose administration and the present results with insulin alone probably reflect increased utilization of carbohydrate.

The effect of insulin on epinephrine-induced changes in other serum lipids is shown in Table 4. It is noteworthy that although serum FFA elevation is blocked in the insulin-treated dogs, there is still a definite effect of epinephrine on serum cholesterol and phospholipids. The elevation in both lipids was, however, less marked when insulin and epinephrine were given as compared with the response of the same dogs treated with epinephrine only.

DISCUSSION

Two distinct lipid-mobilizing activities of epinephrine should be recognized. First, it stimulates the release of free fatty acids (FFA) from tissue depots; second, it causes an elevation of serum lipoprotein levels. It would be important to know whether these two responses are effected through a common primary mechanism of action. It is possible that the FFA responding rapidly to epinephrine may induce in turn the subsequent changes in serum lipoprotein levels. High blood levels of FFA could stimulate lipoprotein synthesis through incorporation of the fatty acid residues into the lipid moieties of the lipoproteins or, alternatively, the increase in circulating lipoproteins might represent a response to the need for an auxiliary FFA carrier protein in addition to albumin. There are a number of clinical situations in which there is an association of high blood levels of both FFA and lipoproteins, notably in uncontrolled diabetes mellitus and starvation (15). In the nephrotic syndrome, where serum albumin concentrations are much below normal, the lipoproteins are elevated and assume a much larger than normal share in blood FFA transport, as shown by Shafrir (16).

The results of the present study, however, suggest that the effects of epinephrine on lipoproteins may in fact not be strictly correlated with its effects on FFA levels. The rise in FFA after epinephrine is marked but short lived. It appears promptly but it cannot be sustained either by continuous infusion of large amounts of saline solutions of the hormone or by the administration of long-acting epinephrine in oil. The FFA level returns to normal before significant elevations in the cholesterol and phospholipid values are noticed. Furthermore, by raising the blood level of glucose prior to the injection of epinephrine or by enhancing glucose utilization by simultaneous insulin

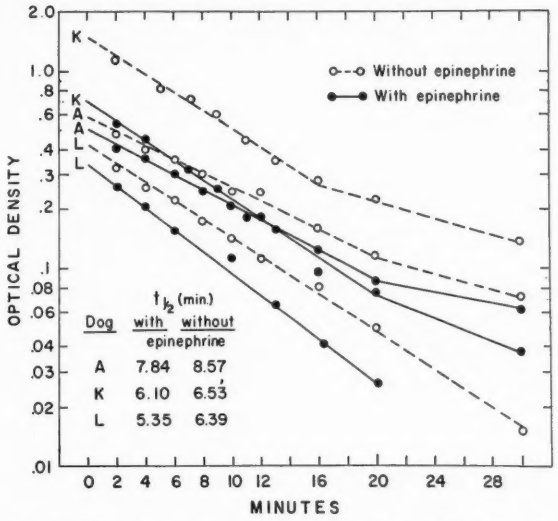

FIG. 5. Chylomicron disappearance curves in three dogs (K, A, and L) with and without prior administration of epinephrine. Epinephrine in oil (1 mg./kg.) was given subcutaneously 30 minutes prior to injection of chylomicrons. Dog K had been given the same dose of epinephrine daily for 2 days and received a third dose on the day of study. Half lives were calculated from the mean slope of the straight line portion of the curves during which more than 90% of the injected chylomicrons disappeared from the circulation

administration, the FFA level can be prevented from rising, and yet this does not prevent the characteristic elevation of cholesterol and phospholipids observed 24 hours after epinephrine. This dissociation of the two responses, however, must be further explored, in particular by studies of the turnovers of FFA and other blood lipids under these conditions.

From the time relationship of the peaks of the serum FFA and glucose curves in normal dogs receiving epinephrine (Figs. 6 and 7), it appears that as the blood glucose level rises, the FFA level falls toward normal. This decline of the FFA level probably is related causally to the increasing level of blood glucose rather than to an exhaustion of the epinephrine effect. Availability of glucose as a calorigenic substrate has been shown by Gordon and by Dole to be a controlling factor in the FFA output of the adipose tissue (17, 18). Moreover, it has been shown by Goldfien and Havel that the FFA response to norepinephrine, which does not produce marked hyperglycemia, is sustained much longer than it is after epinephrine (19).

The serum lipid increment in response to epinephrine appears in the cholesterol- and phospholipid-rich lipoproteins of the 1.019 to 1.063 and 1.063 to 1.21 density class. As noted in Table 3, the percentage rise in the

d = 1.019 to 1.063 fraction was considerably greater than that in the d = 1.063 to 1.21 fraction, a finding compatible with the changes in serum cholesterol to phospholipid ratios with time of epinephrine treatment. Not all these changes, however, can be accounted for on this basis. It appears that the lipid composition of the ultracentrifugal fractions changed significantly, more cholesterol being included in the 1.063 to 1.21 fraction. Further studies of the lipoproteins produced under the influence of epinephrine may be helpful in exploring the limits of variability in lipoprotein synthesis.

The rise in free to total cholesterol ratio at 24 hours may represent rapid cholesterol mobilization from the liver, normally rich in free cholesterol, and the return to the original ratio after 8 days of treatment would represent progressive esterification of this new cholesterol.

Epinephrine caused no rise in the triglyceride-rich d < 1.019 lipoprotein fraction in the present studies. Small or insignificant effects of epinephrine on fasting dog serum triglycerides have been observed when the triglycerides were determined by a direct method of Van Handel and Zilversmit (20).[4] Kaplan *et al.* (3) reported large percentage increases in dog serum triglyceride levels (70 to 100 per cent) following subcutaneous administration of epinephrine in oil. The changes in cholesterol and phospholipid levels were similar to those reported in this communication. It can be seen from Table 1 and Figure 1 that daily fluctuations in serum triglycerides of such an order of magnitude (expressed as percentage changes) are not uncommon and the technical difficulties in the determinations of triglyceride levels have been alluded to above. Moreover, the feeding schedule adopted by Kaplan *et al.* may be relevant since their epinephrine-injected dogs were fed canned meat during the experimental period. In view of the delaying effect of epinephrine on fat absorption described above, it may be possible that the 24-hour increase in the triglyceride levels noted by these investigators represented in part delayed fat absorption.

On the other hand, it appears that there may be a considerable species variation in the nature of the

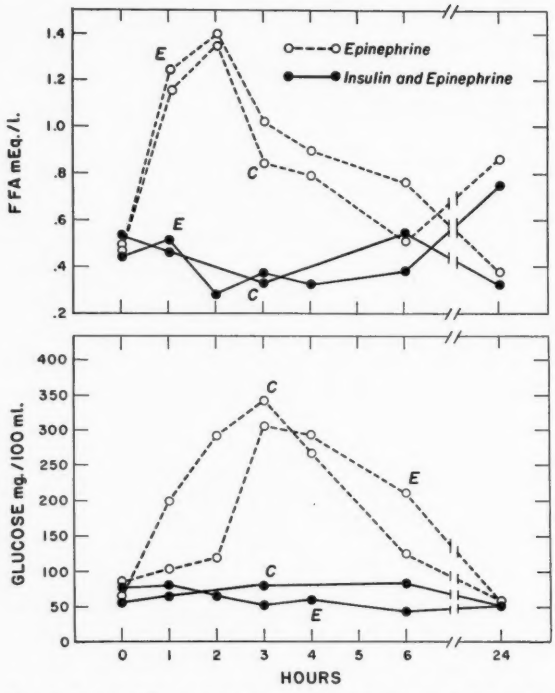

FIG. 7. Serum FFA and glucose levels following administration of insulin with epinephrine. Dogs C and E were injected with insulin and epinephrine at the same time (see Methods). In the control experiments they received epinephrine only.

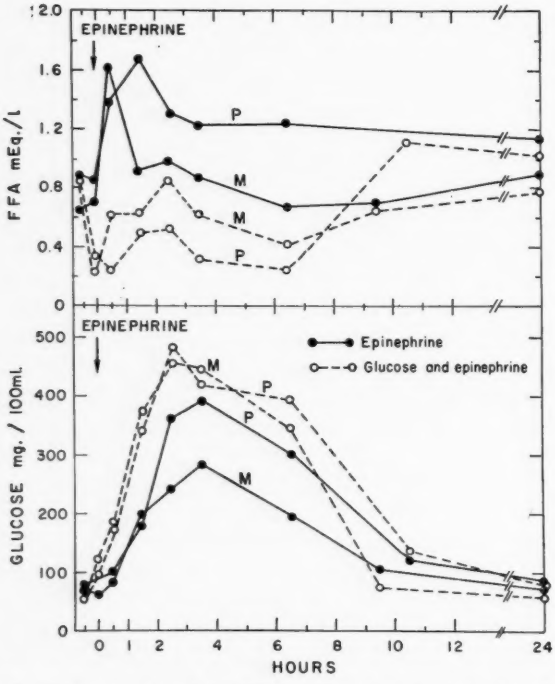

FIG. 6. Suppression of the FFA response to epinephrine by administration of glucose. Glucose solution was given intragastrically ½ hour before epinephrine and continued at ½ hour intervals as indicated in the Methods. In control experiments the same dogs received similar amounts of water along with epinephrine.

[4] W. M. Butler, Jr., Dr. H. Maling, and Dr. B. B. Brodie, personal communication.

hyperlipidemia induced by epinephrine. Dury (4) reported a marked elevation in triglycerides and in low-density lipoproteins in fasting rabbits. But rats do not respond with elevation of triglycerides after long-lasting epinephrine (21). It is pertinent that rabbits, in contrast to dogs, respond with marked triglyceride elevations to treatment with cortisone or to the lack of thyroxine (22).

The possible relationship between the epinephrine-induced hyperlipidemia and the hypercholesterolemia reported by a number of investigators as an accompaniment of prolonged mental and emotional stress (23) and periods of tension (24) may be commented upon. Certainly these reactions entail adrenomedullary activity. Furthermore, the present results indicate that elevated cholesterol levels can be maintained over extended periods under the continuing stimulation of epinephrine. The possibility that the sympathetic nervous system triggers the lipid response directly by way of the adrenal medulla has to be considered along with the possibility of indirect stimulation by way of secondary release of specific pituitary lipid mobilizing factors. More recent studies in this laboratory have shown that both the FFA and the lipoprotein responses to epinephrine are abolished or markedly reduced in adrenalectomized or hypophysectomized rats (21) and dogs (22) and that normal responses to epinephrine are restored by treatment with cortisone. Thus it appears that increased secretion of both the medullary and the cortical hormones of the adrenal may be important in the hypercholesterolemia of the stress (25).

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Independence of arterial phospholipid synthesis from alterations in blood lipids*

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SUMMARY

Rabbits fed cholesterol for 1 to 5 months showed gradually increasing amounts of aortic phospholipid preceded by increases in plasma phospholipid concentration. Cholesterol feeding appeared to increase the turnover of plasma as well as aortic phospholipid. Curtailment of plasma phospholipid synthesis to one-tenth of normal by evisceration did not diminish the incorporation of P^{32} into aortic lipids. In these animals the aortic phospholipid specific activity was 15 to 90 times as great as that of plasma, so that practically all the labeled aortic phospholipid must have been synthesized *in situ*. Lowering the plasma lipid levels by removal of cholesterol from the diet did not diminish the cholesterol content of the aortic lesion or alter its phospholipogenesis.

In previous investigations we have shown that in the cholesterol-fed rabbit, phospholipids accumulate in aortic plaques, and that these phospholipids were probably not deposited from plasma but instead were formed by synthesis in the arterial wall (1). This evidence was obtained by the injection of P^{32}-phosphate and P^{32}-labeled lipoproteins, which demonstrated that over 90 per cent of the radioactive phospholipid in the arterial wall was derived from synthesis. This conclusion was confirmed by the observation that early after P^{32}-phosphate administration, the specific activity of three different phospholipid fractions was greater in the artery than the time-average specific activity of the same phospholipids in plasma (2). Several questions remained to be answered, however. According to our concept that the artery is the source of plaque phospholipids, one should be able to alter the specific activity of the plasma phospholipids drastically without noticeable effect on the incorporation of P^{32} into arterial phospholipids. This was accomplished in the experiments to be reported here by evisceration of the animals prior to P^{32} administration. After demonstrating that the incorporation of P^{32}-phosphate into arterial phospholipid was independent of the level of radioactive plasma phospholipids, it seemed important to find out whether this incorporation would depend on the concentration of nonradioactive lipids in the blood. This question was studied in rabbits with atheromatous lesions and normal blood lipid levels. These studies indicated that the rate of phospholipid synthesis from P^{32} in the atheromatous aorta did not decrease when the plasma lipid levels were lowered. It thus appears that arterial phospholipid synthesis is a process closely related to, if not a part of, the development of experimental atherosclerosis and that the regulation of the synthetic rate is not a simple function of blood lipid concentration.

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† Career investigator, American Heart Association.

EXPERIMENTAL

Experimental atheromatosis was produced in New Zealand white rabbits by daily feeding of 100 g. Purina rabbit chow containing 1 g. cholesterol and 2.6 g. fat.[1] Control animals, which received the same amount of rabbit chow per day, were chosen at the beginning of all feeding periods and were paired with respect to age and source.

Rabbits were injected with 0.5 mc. P^{32}-phosphate and usually plasma, liver, and aorta were taken 4 to 6 hours later for phospholipid P^{31} and P^{32} determination. Some rabbits were killed 6, 24, and 48 hours after P^{32} administration for determination of specific activities of phospholipids in plasma and aorta at various time intervals. To obtain measures of acid-soluble phosphate specific activity in the latter experiment, tissues were first extracted with trichloroacetic acid

[1] Kopald vegetable fat, Humko Co., Memphis, Tenn.

TABLE 1. CONCENTRATION OF PHOSPHOLIPIDS AFTER VARIOUS INTERVALS OF CHOLESTEROL FEEDING

Months	Aorta		Plasma		Liver	
	Control Diet	Cholesterol Diet	Control Diet	Cholesterol Diet	Control Diet	Cholesterol Diet
1	0.160 ± 0.043	0.189 ± 0.059	0.0388 ± 0.0042	0.281 ± 0.071	1.01 ± 0.09	0.889 ± 0.092
2	0.177 ± 0.012	0.372 ± 0.080	0.0442 ± 0.0074	0.252 ± 0.033	1.00 ± 0.12	1.07 ± 0.04
3	0.172 ± 0.022	0.663 ± 0.121	0.0414 ± 0.0068	0.349 ± 0.038	1.30 ± 0.09	1.33 ± 0.06
5	0.147 ± 0.041	0.520 ± 0.046	0.0468 ± 0.0042	0.330 ± 0.031	1.12 ± 0.01	0.995 ± 0.10

Concentrations are expressed as mg. phospholipid P/cc. plasma or/g. fresh tissue. Mean of 3 animals ± standard error.

and then with chloroform-methanol for lipid analysis. Therefore the absolute lipid values in this experiment differed slightly from those obtained when trichloroacetic acid was not used.

In all experiments extraction of tissue lipids was carried out by the method of Folch *et al.* (3). Phospholipid phosphorus in the purified extracts was determined by the method of King (4). Cholesterol was determined on the same extract after saponification and extraction with petroleum ether. To a dried aliquot was added 4 ml. of a solution containing 2.9 per cent p-toluenesulfonic acid, 25.7 per cent glacial acetic acid, and 71.4 per cent acetic anhydride; 30 minutes after addition of 0.4 ml. concentrated H_2SO_4, the optical density was determined at 550 mμ. (5).

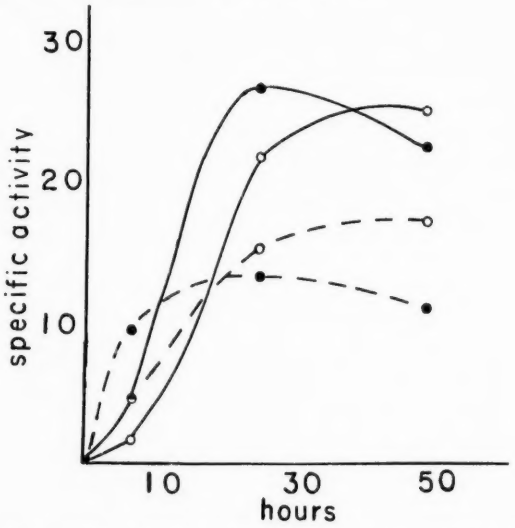

FIG. 1. Specific activity-time relations of plasma (——) and aortic (– – –) phospholipid in control (●) and cholesterol-fed (○) rabbits. Specific activities are expressed as per cent of injected P^{32} per g. of phospholipid phosphorus. Each point represents the mean obtained on 3 animals.

RESULTS

A prerequisite for quantitative isotope studies is the knowledge of specific activity-time relations of the material under study and, if possible, of its immediate precursor. Although some data obtained in our laboratory showed that 6 hours after P^{32} administration the specific activity of aortic phospholipid was still increasing, no literature is available to indicate these specific activity-time relations. Figure 1 shows specific activities of plasma and aortic phospholipids at various times after the injection of P^{32}-phosphate in control and cholesterol-fed rabbits (1 g. cholesterol per day for 5 months). Each point represents the average of duplicate determinations on each of three animals. Although the small number of animals employed per time interval does not allow a detailed interpretation of differences in the specific activity-time course of control and cholesterol-fed rabbits, the data are sufficient to illustrate the following points: (*a*) at the 6-hour interval used in previous studies (6) the specific activity of aortic phospholipid exceeds that in plasma; (*b*) at later intervals the specific activity of plasma rises so rapidly that it soon exceeds that of the aorta; (*c*) the aortic specific activity continues to rise and does not level off until about 24 hours after P^{32} injection. The choice of the 6-hour time interval between injection of P^{32} and killing the animal appears to be appropriate since at this time the specific activity of the plasma still lags behind that of the aorta. This is advantageous for the detection of phospholipid synthesis by the aorta in the presence of a large pool of plasma phospholipid in immediate contact with the artery.

The present study was also concerned with the sequence of phospholipid alterations in plasma and aorta at different intervals after the beginning of a cholesterol-rich diet. Two questions appeared to be of impor-

tance: (*a*) would the increase in arterial plaque phospholipid be preceded by an elevation of plasma phospholipid, and (*b*) would one be able to detect by the P^{32} technique early changes in arterial phospholipid metabolism before any histological or chemical alterations were evident? To study these questions, four groups of three cholesterol-fed and three control rabbits were sacrificed 1, 2, 3, and 5 months after the beginning of the diets. Six hours prior to the terminal samples, 0.5 mc. P^{32}-phosphate was administered intravenously. Table 1 presents phospholipid concentrations in aorta, plasma, and liver in these animals. In confirmation of earlier work (7) it was observed that although the liver was quite fatty, the liver phospholipid concentration per g. of fresh tissue did not increase during the 5-month period. In plasma, major increases in phospholipid concentration became evident after 1 month and in the aorta after 2 months, when the cholesterol-fed animals were compared to their own controls. A study of the plasma and arterial phospholipid concentrations in individual animals revealed that plasma phospholipids rose before any increase in arterial phospholipids occurred. Weinhouse and Hirsch (8) similarly observed that the cholesterol in plasma increases before that in the aorta. This observation and the finding that the lipid partition of the fresh plaque resembles that of the plasma (9) have contributed greatly to the conclusion that plaque lipids are derived from the circulating blood. The P^{32} data in Table 2, in confirmation of earlier observations (6), do not substantiate this conclusion for the phospholipid moiety of the plaque. In the cholesterol-fed animals the specific activity of the acid-soluble phosphates of plasma and aorta did not differ from the controls but the specific activity of arterial phospholipids greatly exceeded that of plasma. If for the moment we ignore the fact that neither the plasma nor the arterial phospholipids are homogeneous, a subject that has been dealt with in a previous publication (2), this difference in phospholipid specific activities would appear to rule out the possibility that a major portion of arterial phospholipids derives from plasma.

Two additional facts emerge from Table 2. The specific activities of phospholipid of control arteries, of arteries with minimal plaques (1 and 2 months) and of arteries with major lipid deposits (3 and 5 months) did not differ appreciably. This indicates that the per cent phospholipid renewed is constant. In other words, per unit of phospholipid complex, be it lipoprotein or a lipid micelle, the turnover is the same in the pathological state as in the normal condition. Since, however, the total thoracic aorta of the severely

TABLE 2. SPECIFIC ACTIVITIES OF PHOSPHOLIPIDS AFTER VARIOUS INTERVALS OF CHOLESTEROL FEEDING 6 HOURS AFTER P^{32} ADMINISTRATION

Months	Cholesterol Diet		Control Diet	
	Aorta	Plasma	Aorta	Plasma
1	6.69 ± 1.88	2.51 ± 0.05	5.84 ± 0.60	5.62 ± 1.64
2	7.65 ± 1.06	1.54 ± 0.19	6.55 ± 3.55	5.11 ± 0.76
3	8.94 ± 2.66	2.62 ± 0.87	7.29 ± 0.69	7.85 ± 2.57
5	7.57 ± 0.37	2.39 ± 0.38	7.24 ± 1.51	4.86 ± 0.69

Specific activities are expressed as per cent injected P^{32}/g. phospholipid P. Mean of 3 animals ± standard error.

atherosclerotic animal contains eight times as much phospholipid as the control artery [2] the total turnover rate of aortic phospholipid was increased by a factor of eight. The other fact which merits discussion concerns the plasma phospholipids. It is evident from Table 2 that the specific activity of plasma phospholipids in the cholesterol-fed animal is lower than in the normal control. One might attempt to translate the depressed plasma phospholipid specific activity in terms of a decrease in plasma phospholipid turnover rate but this would not take into account the fact that the phospholipid concentration of the plasma is increased several-fold. Thus a better measure of turnover rate of plasma phospholipid is the concentration of radioactive plasma phospholipid divided by the liver phospholipid specific activity, since the liver is the primary source of plasma phospholipid.[3] This average ratio is 0.020 ± 0.001 and 0.060 ± 0.004 respectively for the controls and cholesterol-fed rabbits and shows that in the latter the turnover rate of plasma phospholipids is actually increased ($p < 0.001$). One might conclude, therefore, that both plasma and arterial phospholipid turnover per whole organ is greatly increased in the atherosclerotic rabbit and that, as was the case for the concentrations, the increased turnover of plasma

[2] Although Table 1 shows only a fourfold increase in phospholipid concentration, it should be noted that the aorta of the cholesterol-fed animal weighs more than two times that of the control.

[3] Experiments have demonstrated (10) that in the rabbit the removal of the gastrointestinal tract, spleen, liver, and kidneys decreased plasma phospholipid synthesis to approximately one-tenth. Whether the assumption is made that liver is the primary source of plasma phospholipid, or that all of these tissues contribute, does not alter our basic finding of increased plasma phospholipid turnover, as liver, intestine, spleen, and kidney specific activities are of the same order of magnitude and are not altered by cholesterol feeding (7).

TABLE 3. SPECIFIC ACTIVITIES OF TISSUE PHOSPHOLIPIDS IN EVISCERATED-NEPHRECTOMIZED CHOLESTEROL-FED AND SHAM-OPERATED CHOLESTEROL-FED RABBITS 4 HOURS AFTER P^{32} ADMINISTRATION

	Eviscerated-Nephrectomized	Sham-Operated
Liver	0.0242 ± 0.0138	9.59 ± 1.12
Plasma	0.0659 ± 0.0208	0.580 ± 0.099
Intima	4.27 ± 0.68	4.58 ± 0.78
Residual Aorta	5.23 ± 0.56	3.55 ± 0.23

Specific activities are expressed as per cent injected P^{32}/g. phospholipid P. Mean of 3 animals ± standard error.

phospholipids precedes the increase in the artery. However, the isotope data do not support the thesis that plasma phospholipids function as precursors of arterial phospholipids. Unfortunately the P^{32} technique did not measurably increase the sensitivity of detection of early atherosclerotic changes in the arterial wall since changes in P^{32} concentrations did not precede, and were no greater than, changes in chemical phospholipid concentrations.

Although the above experiment confirmed previous evidence (1) that the majority of the arterial phospholipid was synthesized *in situ*, it might still be possible that a very small portion of arterial phospholipid was derived from deposition of blood phospholipid or blood lipoproteins. Therefore the incorporation of P^{32} into arterial phospholipid was studied in animals in which the formation of plasma P^{32}-phospholipids was severely depressed by the removal of gastrointestinal tract, spleen, and kidneys, and the occlusion of the hepatic artery (10). If most of the P^{32}-phospholipid in the atheromatous lesion is derived from synthesis in the artery, one should expect no diminution of arterial phospholipid P^{32} in the face of a greatly diminished plasma phospholipid P^{32}. The results of such an experiment are given in Table 3. In order to work with eviscerated animals before they deteriorated too much, the experiment was terminated 4 hours after injection of P^{32}-phosphate. The extent to which incorporation of P^{32} into liver phospholipids is depressed attests to the success of the hepatectomy. Apparently plasma phospholipid synthesis is practically stopped by evisceration in the cholesterol-fed animal, as in normal controls previously studied (10). The most significant finding was, however, that the incorporation of P^{32} into arterial phospholipids of eviscerated and sham-operated animals was practically the same, although the average plasma phospholipid P^{32} in the eviscerates was only one-tenth that of the sham-operated controls.[4] Stronger evidence that practically all phospholipid P^{32} came from arterial synthesis may be gleaned from a comparison of the terminal specific activities of aortic and plasma phospholipids in individual eviscerated cholesterol-fed rabbits, as in Table 4. In some of the eviscerates plasma phospholipid-P^{32} incorporation was not so greatly depressed as in others. In one animal, however, the ratio of aortic to terminal plasma phospholipid specific activity reached 93.8, which is clear evidence that the P^{32}-phospholipid was derived entirely from aortic synthesis.

From the above experiments no evidence could be obtained to suggest that deposition of plasma phospholipids is responsible for the incorporation of P^{32} into the phospholipids of arterial plaques. Yet the question whether plasma lipids are causally linked in some other manner to the development of arterial lesions cannot be resolved by these experiments. Perhaps a high concentration of plasma cholesterol or plasma phospholipid is required to bring about the accumulation of arterial phospholipids by means of an increase in phospholipid synthesis. We have attempted to answer this question by comparing the synthesis of phospholipids in atheromatous lesions of hyper- and normolipemic animals. The latter were prepared by feeding 1 g. of cholesterol per day for 3 months and subsequent removal of dietary cholesterol for 2 months. During the latter period the blood lipids de-

TABLE 4. COMPARISON OF TERMINAL PHOSPHOLIPID SPECIFIC ACTIVITIES IN INDIVIDUAL EVISCERATED-NEPHRECTOMIZED CHOLESTEROL-FED RABBITS

Aortic Intima	Plasma	$\frac{\text{Aortic Intima}}{\text{Plasma}}$
4.01	0.208	19.3
2.53	0.190	13.3
6.05	0.284	21.3
3.85	0.062	62.1
5.50	0.099	55.5
3.47	0.037	93.8

Specific activities are expressed as per cent injected P^{32}/g. phospholipid P.

[4] One might expect to find an increased specific activity in both lesions and residual aorta of the eviscerated-nephrectomized rabbit resulting from a decreased precursor pool which was reflected in a terminal plasma acid-soluble phosphate specific activity of 301 ± 74 compared to 91.9 ± 6.8 in the controls. However, the fact that the intima showed no such difference might be explained on two grounds: (*a*) the small number of animals in this experiment and the presence of wide fluctuations, and (*b*) the 20 per cent greater phospholipid content of the lesions in the eviscerated-nephrectomized group.

TABLE 5. PHOSPHOLIPID AND CHOLESTEROL CONCENTRATIONS IN TISSUES OF HYPERLIPEMIC AND NORMOLIPEMIC RABBITS—EXPERIMENT 1

	Phospholipids		Cholesterol	
	Hyperlipemic	Normolipemic	Hyperlipemic	Normolipemic
Liver	33.0 ± 3.0	32.8 ± 3.0	58.5 ± 12.6	5.61 ± 1.18
Plasma	8.32 ± 0.14	1.25 ± 0.09	21.4 ± 2.5	1.23 ± 0.32
Intima	25.2 ± 3.8	21.4 ± 2.6	81.0 ± 13.3	59.6 ± 5.0
Residual Aorta	9.90 ± 0.68	6.38 ± 0.35	20.5 ± 2.1	11.4 ± 1.3

Concentrations are expressed as mg./g. fresh tissue or /cc. plasma. Mean values of 4 animals ± standard errors.

creased to almost normal values but the atheromas persisted. These animals were compared to another group of four animals who were simultaneously maintained on rabbit chow for 2 months and subsequently on chow plus cholesterol for 3 months. Table 5 presents the phospholipid and cholesterol content of liver, plasma, aortic intima, and residual aorta (media and adventitia). Strictly speaking, a comparison of the extent of the lesions in the two groups might be misleading since the period of hypercholesterolemia in the normolipemic group was longer than in the hyperlipemic group. This is due to the fact that high serum cholesterol values persist after removal of the dietary cholesterol. To control this factor we maintained a second group of six animals on a high cholesterol diet for 3 months followed by a 2-month period on Purina chow (normolipemic group as above), six rabbits on Purina chow for 1 month, followed by 4 months on 1 per cent cholesterol diet (hyperlipemic group), and six animals on Purina chow for the entire 5 months. The course of serum cholesterol levels of these three groups is presented in Figure 2. In agreement with observations of McMillan *et al.* (11) and Friedman and Byers (12), we found that serum cholesterol levels decreased gradually over a period of 2 months after cessation of cholesterol feeding, and were almost down to normal at the end of 2 months. Although the hyperlipemic group continued on a high cholesterol diet, it also showed an appreciable decrease of serum cholesterol levels after the first month but the levels plateaued at around 1000 mg. per 100 ml. A similar phenomenon has been reported previously by several workers (8, 13) after 3 months of cholesterol feeding. Table 6 lists the concentrations of phospholipids and cholesterol of liver, plasma, aortic intima, and residual aorta, as in Table 5. Here again the liver cholesterol concentrations had decreased markedly in the normolipemic group although they did not quite reach the values of normal controls. It is interesting that the liver weights of the normolipemic as well as the hyperlipemic animals were about twice those of the control animals, although body weights were not significantly different. Similarly, the weights of the thoracic aortas in all cholesterol-fed rabbits exceeded those of the controls. In the hyperlipemic group the mean aortic weight was 1.06 with a standard error of 0.11 g. (intimal weight 0.46 ± 0.06), in the normolipemic animals the weight averaged 1.29± 0.12 g. (intimal weight 0.62 ± 0.05), and in the controls only 0.52 ± 0.04 g. Thus the concentrations of cholesterol and phospholipids in the atherosclerotic arteries do not give a true index of the degree of accumulation of lipids in these organs. The residual aorta (media and adventitia) also showed an increase in cholesterol and phospho-

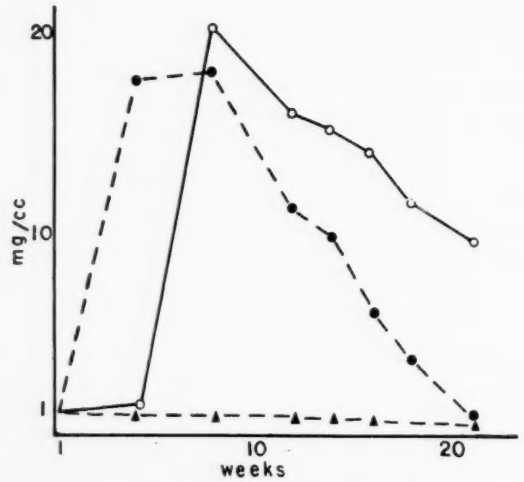

FIG. 2. Plasma cholesterol concentrations in control (--▲--), normolipemic (--●--) and hyperlipemic (—○—) rabbits at different intervals. Each point represents the mean of 5 or 6 animals. (Experiment 2, see text.)

TABLE 6. PHOSPHOLIPID AND CHOLESTEROL CONCENTRATIONS IN TISSUES OF HYPERLIPEMIC, NORMOLIPEMIC, AND CONTROL RABBITS—EXPERIMENT 2

	Phospholipids			Cholesterol		
No. of Animals	Control 5	Hyperlipemic 6	Normolipemic 5	Control 5	Hyperlipemic 6	Normolipemic 5
Liver	31.0 ± 1.8	27.0 ± 1.0	25.0 ± 1.0	2.19 ± 0.12	30.2 ± 4.27	8.20 ± 1.96
Plasma	0.728 ± 0.117	4.02 ± 0.71	1.18 ± 0.156	0.362 ± 0.053	7.91 ± 1.85	0.606 ± 0.330
Intima		27.5 ± 8.4	25.0 ± 3.8		64.3 ± 8.7	45.6 ± 9.5
Residual Aorta	5.12 ± 0.26	8.22 ± 0.97	8.42 ± 2.34	1.96 ± 0.47	11.6 ± 2.0	6.90 ± 1.80

Concentrations are expressed as mg./g. fresh tissue or /cc. plasma. Mean values ± standard errors.

lipid concentration, but it is difficult to tell whether this increase is the result of aortic tissue lipid or of lipid adhering to the adventitia. When one compares the total aortic cholesterol or phospholipid of the normolipemic groups in both experiments with that of the hyperlipemic groups, it is evident that no appreciable regression of the lesions had taken place as far as their lipid content is concerned.

In both experiments all animals received 0.5 mc. P^{32}-phosphate intravenously and 6 hours later were sacrificed for chemical and radioactive analyses. As there was no detectable difference in the data obtained from experiments 1 and 2, the radioactive analyses are presented in combined form in Table 7. As discussed previously in this paper, the specific activity of aortic phospholipids does not differ greatly in animals with severe lesions and those with few or no lesions. Therefore to get a better index of the rate of synthesis of phospholipids per aorta, the incorporations of injected P^{32} per whole thoracic aorta in the control, hyperlipemic, and normolipemic animals are compared. As before, we found that the incorporation of P^{32} in hypercholesterolemic animals is about six times greater than in normal controls. It is interesting, however, in this experiment that in those animals in which the blood lipids were allowed to drop to nearly normal values, the rate of incorporation of radioactive phosphate into phospholipids of the atheromatous aorta stayed at a level five times above normal and was not significantly different from the rate observed in the hyperlipemic animals. Apparently the rate at which P^{32} is incorporated into aortic phospholipids does not depend on the extent of serum lipid elevation.

DISCUSSION

From the above experiments no evidence could be obtained to suggest that deposition of plasma phospholipids is responsible for the incorporation of P^{32} into the phospholipids of arterial plaques. Previous studies with P^{32}-labeled lipoprotein also indicated that most of the radioactive phospholipid in the lesion was de-

TABLE 7. RADIOACTIVE DATA FROM HYPERLIPEMIC-NORMOLIPEMIC RABBITS (EXPERIMENTS 1 AND 2)

	Number of Animals	Specific Activity			Per Cent Injected Dose per Total Aorta × 10^3	
		Plasma	Aortic Intima	Residual Aorta	Aortic Intima	Residual Aorta
Control	5	5.10 ± 0.85		4.45 ± 0.69 *		0.461 ± 0.059 *
Hyperlipemic	10	2.19 ± 0.33	6.00 ± 0.75	6.29 ± 1.21	2.92 ± 0.71	1.09 ± 0.12
Normolipemic	9	3.65 ± 0.43	4.48 ± 0.50	4.30 ± 0.81	2.45 ± 0.28	0.528 ± 0.077

* Whole aortic wall, since separation of intima in control animals proved impractical. Phospholipid specific activity is expressed as per cent injected P^{32}/g. phospholipid P. Mean ± standard error.

rived from synthesis *in situ* (1). Although this evidence strongly suggests that all the excess phospholipid in the atheroma is derived from local synthesis, such a conclusion cannot be asserted with absolute certainty. A rough calculation of the rate of accumulation of arterial phospholipid during the period of cholesterol feeding shows that deposition of as little as 5 μg. of phospholipid P per hour could account for the phospholipid in the plaque. This rate is well below the sensitivity of our tracer measurements. Furthermore, our studies pertain only to the phosphorus portion of the phospholipid molecule. It is possible that phospholipids arrive at the intima by transport from plasma and that the phosphorus portion exchanges rapidly with the phosphorus pool of the intima. Studies with labeled fatty acids might yield additional evidence on this point. Should one therefore wish to preserve the idea that plaque phospholipids are derived from the deposition of plasma lipid or lipoprotein, our experiments could not strictly reject such a hypothesis. On the other hand, it would appear that the deposition hypothesis, for phospholipids at least, is based in part on analogies with the behavior of cholesterol. Even for the latter substance direct proof of lipid deposition is unavailable to date, although suggestive evidence has been presented. In the absence, then, of good reasons for assuming phospholipid deposition and with the availability of evidence for accelerated production of phospholipids in the lipid-laden intima, it would appear that the concept that all excess phospholipid in the atheroma is derived from synthesis *in situ* is a reasonable working hypothesis.

The implications of such a hypothesis are manifold. One should like to know whether the phospholipids in the atheroma are part of the initial injury to the artery or whether they might serve to protect the arterial intima from cholesterol accumulation. The well-known surface active properties of phospholipids would appear to lend credence to the speculation that the accelerated turnover of intimal phospholipids aids in the removal of cholesterol by solubilization of the latter from the arterial wall into the plasma. Such action would appear to be specific to the atheroma since in other tissues, including the cholesterol-rich liver, no increase in phospholipid P^{31} and P^{32} concentration is observed. This should not be too surprising, however, in view of the fact that the liver can dispose of cholesterol by excretion of the sterol itself or its derivative, bile acid. Moreover, the solubilization of liver cholesterol by excess liver phospholipid synthesis would increase the serum cholesterol level, which would presumably not enhance the survival of the animal. The aorta, on the other hand, does not have an excretory mechanism like the liver, and unless cholesterol can be oxidized by arteries (about which no data are available), solubilization of cholesterol might be the only mechanism whereby the artery could rid itself of excess cholesterol. To what extent these findings and speculations are applicable to human arterial disease is not known at the present time and must await further metabolic work on human arterial tissues.

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Liver lipid response to intravenous rare earths in rats

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SUMMARY

Intravenous injection of rare earths with low atomic numbers produces fatty infiltration in the liver of rats. This infiltration is maximal 48 hours later and is characterized by an increase in neutral fat esters. Total cholesterol and phospholipids of the liver in cerium-treated rats were similar to those of control livers. Cerium was found to be effective in producing extremely high levels of liver fat in female rats of several strains tested. Male rats treated in the same way did not exhibit the consistent response or the high liver-lipid levels that were seen in the females. After the castration of male rats, their response resembled that of females. Testosterone caused a significant reduction in the fatty infiltration induced by cerium in both intact and ovariectomized females. Hypophysectomy prevented fatty livers in both females and males, whereas adrenalectomy did so only in males. Choline and methionine had no protective effect against cerium. Tracer experiments involving cerium^{144} demonstrated that most of the radioactivity appeared in the acid-soluble fraction of the liver and that the lipid fraction contained essentially no tracer.

The group of elements known as the rare earths has received relatively little attention in biological systems. The ability of these metals to behave like colloids at physiological pH has suggested some therapeutic possibilities (1), their increased industrial use (2) stresses the importance of acquiring a better biochemical understanding of this group of elements.

In a series of experiments designed to evaluate the clinical usefulness of different rare earths, a conspicuous blanching was observed in the livers of rats injected with cerium. Additional work showed that this blanching was the result of fatty infiltration (3). The initial studies showed levels of total liver lipid to be as high as 15 per cent within 48 hours after administration of cerium. We believe that no chemical or dietary means so far shown to cause fatty livers can do so as rapidly and extensively as a single injection of cerium. It appears that some of the rare earths would be useful experimental tools in the study of lipid metabolism.

This report represents the first detailed study of the various factors that influence development of the cerium-induced fatty liver. The data show the effect of dosage, time, endocrines, and lipotropic agents on the development of this condition. The data also demonstrate that the fatty infiltration caused by rare-earth elements of low atomic number is characterized by an increase in the content of esterified neutral fat.

METHODS

Animals. Male and female rats of the Sprague-Dawley (S-D), Charles River (CR), Carworth Farms Nelson (CFN), and Wistar (W) strains weighing 180 to 250 g. were maintained on Rockland rat pellets. Water was available at all times. Hypophysectomized animals were obtained from the Charles River Breeding Laboratories, but gonadectomies and adrenalectomies were performed by the usual surgical procedure in our laboratory. The success of each adrenalectomy was determined by the water tolerance test of Beatty *et al.* (4).

Preparation of Rare-Earth Solutions. All stock solutions were prepared quantitatively from the corresponding oxides (5). The procedure consisted of dissolving the oxides, with the exception of that of cerium, in either hydrochloric or nitric acid. The difficulty encountered with cerium was circumvented by using the following modified method (6). Equal weights of cerium oxide and sodium iodide were boiled vigorously in concentrated hydrochloric acid until the

TABLE 1. EFFECT OF CERIUM DOSE ON SURVIVAL TIME AND TOTAL LIVER LIPIDS IN CFN MALE AND FEMALE RATS

Cerium Administered	Sex	Total Liver Lipids in Wet Tissue		
mg./kg. body wt.		*per cent ± S.D. 24 hours*	*per cent ± S.D. 48 hours*	*per cent ± S.D. 72 hours*
0.0	F		6.11 ± 0.94 (7/7)	
0.5	F		6.60 ± 0.47 (3/3)	
1.0	F	8.10 ± 1.47 (3/3) *	11.69 ± 2.98 (3/3)	8.19 ± 2.27 (3/3)
2.0	F	10.23 ± 1.13 (3/3)	15.82 ± 1.30 (3/3)	17.17 (1/3)
3.5	F	9.77 ± 0.87 (3/3)	11.67 ± 0.43 (2/3)	(0/3)
4.5	F	9.93 ± 2.24 (3/3)	14.24 ± 1.90 (2/3)	(0/3)
0.0	M		5.34 ± 0.33 (3/3)	
3.5	M		7.93 ± 2.43 (3/3)	
7.0	M		9.54 ± 1.04 (3/3)	
14.0	M		9.16 ± 1.04 (3/3)	

* The ratio in parenthesis indicates (number of rats surviving/number of rats receiving dose).

cerium was dissolved and most of the iodine was evolved. Sodium nitrite was added and the mixture was heated again to ensure the complete removal of the iodine from the solution. The acid was removed by gentle evaporation and the residue was dissolved in 6 N HCl. Various dilutions of the stock rare-earth solutions, adjusted to pH 4, were used for all animal injections.

Injection of Rare Earths. Unless otherwise stated, 3.5 mg. of element per kilogram of body weight were injected into the tail vein. The volume of rare-earth solution administered was between 0.1 and 0.5 ml. per rat depending on the body weight of the rat and on the rare-earth solution used. Control animals were injected in the same way with an equivalent volume of saline. The following rare earths were used: cerium, lanthanum, praseodymium, neodymium, samarium, gadolinium, dysprosium, holmium, lutetium, and yttrium. To determine the distribution of cerium in the liver at the peak of fatty infiltration, two rats received 12 microcuries of cerium^{144} intravenously.[1]

Treatment of Animals. One group of animals received intramuscular injections of testosterone propionate or estrone (0.2 mg. in 0.1 ml. saline per rat per day for 16 days). To evaluate the protective effect of lipotropic agents, one group of rats received choline (100 mg. per kg. body weight, intraperitoneally) 1 hour before and 24 hours after the cerium injection; another group received methionine (7 mg. per kg. body weight, intravenously) 5 minutes before the cerium injection.

Analytical Procedures. The rats were killed by decapitation at various periods (usually 48 hours) after injection of the rare-earth solution. A 3-g. portion of the liver was removed, weighed, covered with 95 per cent ethanol, minced, and allowed to dehydrate overnight. The sample was transferred to a glass extraction thimble and the lipids were extracted for 4 hours with a 2:1 (v/v) ethanol:ether mixture in Soxhlet continuous extractors. The combined ethanol and ethanol:ether extracts were evaporated under infrared lamps and extracted with chloroform. Total lipids were determined gravimetrically on aliquots of the chloroform solution. The quantity of lipid present was expressed as percentage of the wet tissue weight, because the moisture content of the liver was not significantly altered by the rare-earth treatment. The per cent water in three control and five cerium-treated livers was 72.4 ± 0.8 and 72.9 ± 1.8, respectively. Ester equivalents (7) were measured in the neutral and phospholipid fractions, which had been separated from each other on a silicic acid column; chloroform and methanol were used as eluting solvents, essentially according to the method of Borgström (8). Mallinckrodt AR silicic acid (100 mesh) was activated by drying at 110°C for 24 hours before use. Phospholipid phosphorus was determined on all fractions by the method of King (9), using Elon as the reducing agent (a substitute for aminonaphtholsulfonic acid), prepared according to Harris and Popat (10). The method of Nieft and Deuel was adapted with slight modification for the measurement of total cholesterol (11) in the neutral fat fractions.

The livers of the rats that received cerium^{144} were separated into trichloracetic acid (TCA) soluble, lipid, and protein fractions. Weighed portions of the

[1] Cerium^{144} obtained from the Oak Ridge National Laboratory.

liver were homogenized in cold 10 per cent TCA containing 0.4 M $MgCl_2$ (12), centrifuged at 4°C, and the supernatant (acid-soluble fraction) was decanted. The residue was washed twice with the TCA solution and the supernatants were combined with the acid-soluble fraction. The lipids in the residue were extracted as described previously. The lipid-free residue, designated as the protein fraction, was dissolved in a boiling HNO_3:HCl (3:1 v/v) acid mixture. Aliquots of the various fractions were radioassayed with a scintillation detector (Nuclear-Chicago). The results were calculated as percentage of total radioactivity in the liver.

RESULTS

Table 1 shows the effects of dose and time after intravenous injection of cerium on the development of fatty livers and on survival of CFN rats. Only the total lipids of livers from survivors is given in the table. For female rats the maximum fat values usually appear at 48 hours after injection, and a dose as low as 1.0 mg. causes a significant increase (13) ($p < 0.01$) in liver lipids within 24 hours. Mortality increases with dose and with time. Because of less response and greater tolerance to cerium by male rats than by female rats, doses up to 14 mg. per kg. were used in four groups of males and all the animals survived. Liver lipids were significantly elevated by 7 and by 14 mg. of cerium per kilogram when compared with the saline group ($p < 0.01$) but no significant difference occurred among the three treated groups.

Variability in the fatty-liver response of male S-D rats to cerium in some early experiments prompted a series of tests in which the fatty-liver response in several strains of females and males was studied. Table 2 demonstrates that the degree of cerium-induced fatty liver is definitely sex dependent. Males are less responsive than females to fatty infiltration in all the strains that were compared. S-D and CFN males showed little or none of this response to cerium, whereas males of the W and CR strains showed an intermediate but significant response.

When castration of S-D rats preceded the cerium treatment, a lipid response similar to that of females was observed (Table 3). On the other hand, testosterone caused a significant reduction in liver fat in both intact and in ovariectomized females. Estrogen, when administered to castrated males, had no additional effect on the liver-lipid response to cerium.

In an attempt to see whether a parallelism exists to other types of chemically induced fatty livers, the role of the adrenals and of the pituitary on the fatty infiltration was examined. By comparison with Table 2, Table 3 indicates that adrenalectomy prevents fatty liver infiltration due to cerium in male rats of both the S-D and CR strains, whereas adrenalectomy only slightly reduces the fatty infiltration due to cerium in female rats. A similar comparison with intact animals shows that hypophysectomy prevents the occurrence of fat accumulation due to cerium in both male and female rats (Table 3).

Table 4 demonstrates that neither choline nor methionine has any protective effect against the fatty liver caused by cerium under the conditions of our experiments.

A survey of the ability of other rare earths to cause fatty livers is reported in Table 5. The data show that the fatty infiltration caused by rare earths is a biological property of the first third (At. No. 57 to 62) of the elements in this series, commonly called the cerium group of rare earths.

Silicic acid fractionation of the total lipids obtained from rats injected with rare earths showed significant increases in neutral lipid ester equivalents when compared with those of the controls (Table 6), whereas the levels of cholesterol and phospholipid in livers of cerium-treated animals were similar to controls. A more detailed study of the specific lipid components in these fractions is in progress.

TABLE 2. TOTAL LIVER LIPIDS OF MALE AND FEMALE RATS OF DIFFERENT STRAINS 48 HOURS AFTER INTRAVENOUS INJECTION OF CERIUM

Cerium Administered	Strain	Sex	Number of Rats	Total Liver Lipids in Wet Tissue	Probability *
mg./kg. body wt.				*per cent ± S.D.*	
0	S-D	M	13	4.72 ± 0.75	
3.5	S-D	M	14	6.23 ± 2.21	
0	S-D	F	2	6.25 ± 0.16	
3.5	S-D	F	15	12.39 ± 1.26	<0.01
0	CR	M	8	5.60 ± 0.71	
3.5	CR	M	7	9.71 ± 2.22	<0.01
0	CR	F	5	6.32 ± 0.80	
3.5	CR	F	6	12.83 ± 2.52	<0.01
0	CFN	M	6	5.30 ± 1.08	
2	CFN	M	6	5.90 ± 1.76	
0	CFN	F	7	6.11 ± 0.94	
3.5	CFN	F	12	11.35 ± 1.84	<0.01
0	W	M	3	4.64 ± 0.25	
3.5	W	M	4	10.49 ± 2.62	<0.02

* The probability that the difference between the observed mean and the saline control for each group is due to chance.

TABLE 3. EFFECT OF GONADECTOMY, ADRENALECTOMY, AND HYPOPHYSECTOMY ON TOTAL LIVER LIPIDS 48 HOURS AFTER INTRAVENOUS INJECTION OF CERIUM

Cerium Administered	Strain	Sex	Number of Rats	Total Liver Lipids in Wet Tissue	Probability [a]
mg./kg. body wt.				*per cent ± S.D.*	
			Intact		
0	S-D	M	13	4.72 ± 0.75	
3.5	S-D	M	14	6.23 ± 2.21	
			Castrated		
0	S-D	M	4	5.73 ± 0.43	
3.5	S-D	M	7	10.01 ± 1.22	<0.01*
3.5	S-D #	M	7	10.12 ± 1.35	<0.01****
			Intact		
0	S-D	F	2	6.25 ± 0.16	
3.5	S-D	F	15	12.39 ± 1.26	<0.01****
3.5	S-D ##	F	10	10.99 ± 1.19	<0.02**
			Ovariectomized		
0	CFN	F	8	7.20 ± 0.67	
3.5	CFN	F	8	14.93 ± 1.84	<0.01****
3.5	CFN ##	F	8	11.61 ± 1.28	<0.001***
			Adrenalectomized		
0	CFN	F	4	5.68 ± 0.19	
3.5	CFN	F	5	11.95 ± 1.16	<0.01*
0	S-D	M	4	4.11 ± 0.32	
3.5	S-D	M	5	4.37 ± 0.48	
0	CR	M	5	4.50 ± 0.30	
3.5	CR	M	2	4.33 ± 0.58	
3.5	CR	F	4	9.09 ± 2.88	
			Hypophysectomized		
0	CR	M	5	4.93 ± 0.50	
3.5	CR	M	3	5.54 ± 0.29	
0	CR	F	7	5.03 ± 0.32	
3.5	CR	F	6	5.34 ± 0.32	

[a] The probability that the difference is due to chance between: the observed mean and the saline (intact or castrated) or cerium control*; the observed mean and the cerium control**; the cerium ovariectomized and the saline control***; and the observed mean and the saline control****.

0.2 mg. estrone or ## testosterone injected intramuscularly per rat per day.

TABLE 4. EFFECT OF CHOLINE OR METHIONINE ON LIVER FAT DEPOSITION DUE TO CERIUM IN FEMALE CFN RATS

Cerium Administered	Treatment	Number of Rats	Total Liver Lipids in Wet Tissue
mg./kg. body wt.			*per cent ± S.D.*
0	None	7	6.11 ± 0.94
3.5	None	5	11.70 ± 0.90
3.5	Methionine *	5	13.32 ± 2.23
3.5	Choline †	4	10.41 ± 1.01

* 7.0 mg./kg. injected intravenously 5 minutes before cerium injection.

† 100 mg./kg. injected intraperitoneally 1 hour before and 24 hours after cerium injection.

The distribution of cerium^{144} in the TCA-soluble, lipid, and protein fractions of the liver at the peak of fatty infiltration was 94.0, 0.1, and 5.9 per cent, respectively.

DISCUSSION

The intravenous administration of rare earths with low atomic numbers (57 to 62) shows a pronounced effect on fat metabolism in the rat. Investigations of respiration by liver slices [2] together with studies of toxicity (5) and distribution (14) have indicated that the heavier rare earths behave more like yttrium and the lighter rare earths more like lanthanum. This is borne out by the liver-fat values for yttrium and lanthanum with respect to those shown for the other rare earths used (Table 5).

The difference we found between male and female rats in the liver-lipid response is also illustrated in reports by other investigators who have used ethionine (15, 16), ethanol (17), and low protein diets (18), rather than cerium, to produce fatty livers. Our data demonstrate that testosterone is definitely a causative factor in this sexual difference. Hormonal influence on lipid metabolism has not been clearly defined (19, 20) but the recently reported *in vitro* studies of Perry and Bowen (21) indicate that the gonads definitely have a role in the biosynthesis of lipids. These workers have demonstrated that castration in the male, but not in the female, is followed by an increased incorporation of 2-C^{14}-acetate into the fatty acids of liver and adipose tissue. Testosterone treatment in the male and estrogen treatment in the female resulted in the decrease and in the increase, respectively, of the incorporation of acetate into liver lipids. Clear-cut interpretation of other data of this type is often made difficult by the complicated pituitary-adrenal-gonad relationships (22).

In the light of known lipid metabolism, the inability of the lipotropic agents to protect against cerium-induced fatty livers indicates that the rare-earth type of fatty liver is nonspecific. The molar ratios of choline:cerium (33:1) and of methionine:cerium (2:1) were such that the quantity of lipotropic agents was well in excess of that of cerium. Methionine has been shown to protect against the ethionine-induced fatty liver even when the molar ratio of methionine to ethionine was 1:4 (16).

The fatty liver induced by rare earths is similar to the fatty infiltration caused by ethanol (23, 24) in that hypophysectomy and adrenalectomy prevent the deposition of fat. Other types of "nonspecific" fatty livers, e.g., phosphorus (25), are also similar in this respect. Some workers have implied that hormones of the pituitary exhibit lipid mobilizing properties (26). Mallov and Bloch (24) have reported that pituitary tissue homogenized in saline, when injected intravenously, can cause a significant increase in total liver fat, but we could not demonstrate any such influence on lipid metabolism under supposedly identical experimental conditions.

Experiments ruled out the possibility that a reduced food intake after the rare-earth injection and fasting for 24 hours before killing might cause some of the observed fatty infiltration. Total liver lipids in two rats fasted as long as 72 hours showed values similar to those of control animals (6.34 and 7.11 per cent).

A considerable effort has been expended in these laboratories in studying the distribution of cerium when administered intravenously to dogs, although very little work has been done in the rat. Our studies indicate that most of the cerium in the rat liver is associated with the TCA-soluble fraction, but we believe that the high cerium^{144} activity in the acid-soluble fraction is a result of solubilization of cerium by the low pH of the TCA solution. Other related studies in our laboratories have suggested the ability of rare earths to complex with proteins at physiological pH. The fact that the lipids contain no radioactivity seemingly rules out the possibility that any strong cerium chelate complex is formed between fatty acid carboxyl and phosphate hydroxyl groups in lecithin or other conjugated lipids. If such a complex were formed, fatty acid transfer from the liver via phospholipids might be seriously impaired. That such complexes are not formed *in vivo* and then dis-

[2] A separate report by E. A. Cress and G. C. Kyker, in preparation.

TABLE 5. TOTAL LIVER LIPIDS OF CFN FEMALE RATS 48 HOURS AFTER INTRAVENOUS INJECTION OF DIFFERENT RARE EARTHS

Rare Earth	Dose Administered	Number of Rats	Total Liver Lipids in Wet Tissue	Probability *
	mg./kg. body wt.		*per cent ± S.D.*	
None	0	7	6.11 ± 0.94	
Lanthanum	2	2	12.43 ± 0.92	<0.001
Cerium	2	3	15.82 ± 1.30	<0.001
Praseodymium	2	4	14.70 ± 1.40	<0.001
Neodymium	3.5	3	14.10 ± 1.45	<0.001
Samarium	3.5	3	12.53 ± 1.57	<0.001
Gadolinium	3.5	3	5.47 ± 0.65	
Dysprosium	3.5	3	6.57 ± 0.71	
Holmium	3.5	3	6.44 ± 0.94	
Lutetium	3.5	3	5.74 ± 0.51	
Yttrium †	3.3	2	5.01 ± 0.40	
Yttrium †	12.5	2	4.33 ± 0.31	

* The probability that the difference between the observed mean and the saline control for each group is due to chance.

† Animals were sacrificed 24 hours after injection of yttrium.

sociated by the strong acid during fractionation gains support by the absence of radioactivity in the total lipid fraction of dog liver, which had not been subjected to TCA extraction after an intravenous dose of tagged cerium.[3]

It is of interest to note that the elevated lipid level in rat livers is a result of glyceride infiltration. At the time when there was a maximal fatty-liver response, the phospholipid and cholesterol content of the livers remained essentially unaltered. The high ester-bond to phosphorus ratio is difficult to interpret. The methods were validated by theoretical results on several purified compounds and a commercial sample of lecithin purified by the same silicic acid method gave an ester to phosphorus ratio of 2.05; also, a commercial preparation without further purification showed a ratio of 1.88. Marinetti *et al.* (27) report an unidentified phospholipid fraction, with phosphatidic acid properties, that gave an unexplained high ester to phosphorus ratio of 3.58; they also used a silicic acid column to separate this fraction from rat liver lipids. The phospholipid fraction separated under our conditions represents a complex mixture of naturally occurring polar lipids. Proteolipids or sulfatides might possibly explain the high ratio, since Gaitonde and Richter (28, 29) have detected small but significant amounts of sulfur-containing lipoproteins in lipid extracts of rat liver. Any contaminating neutral lipid

TABLE 6. SEPARATION OF LIVER LIPIDS INTO NEUTRAL AND PHOSPHOLIPID FRACTIONS ON SILICIC ACID

Treatment	Number of Rats	Total Liver Lipids in Wet Tissue	Neutral Lipid per Gram Wet Tissue		Phospholipid per Gram Wet Tissue	
			Ester	Cholesterol	Ester	Phosphorus
		per cent ± S.D.	*μ eq. ± S.D.*	*mg. ± S.D.*	*μ eq. ± S.D.*	*μ eq. ± S.D.*
Saline control	3	5.63 ±0.20	67.7 ±19.7	3.26 ±0.30	103.1 ±14.2	34.6 ±8.5
Cerium-treated (2 mg./kg. body wt.)	3	15.33 ±2.50	432.5 ±82.2	3.14 ±0.92	94.1 ±14.0	36.0 ±2.3

[3] Unpublished data.

was ruled out by control experiments and the absence of any ester-positive material in the last 28 ml. of chloroform (four-column fractions) prior to the methanol elution of the phospholipids. There is also the possibility of some change during extraction or chromatography that would result in a loss of phosphorus but this seems highly improbable to us.

Experiments are currently under way to determine to what extent mobilization or biosynthesis or both account for the enormous influx of glycerides in the liver of rats treated with cerium. Also the early results of other studies of fatty infiltration suggest quite a different response to cerium in various species.

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Notes on Methodology

A calculation for locating the carrier gas front of a gas-liquid chromatogram

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► The linear relationship between the logarithm of the reduced retention volume (V_R') and the chain length (n) of a series of structural homologues is a feature of gas chromatography which facilitates the identification of components (1). To determine V_R' one must establish the time of appearance of the carrier gas front. In an ideal system this point is considered to be the moment when an infinitesimal amount of nonabsorbed gas introduced with the sample moves through the detector under over-all conditions of constant temperature and pressure gradient. James and Martin (1) designated the air peak as the carrier gas front, and the retention time of a component was obtained directly by measuring from this air peak to the peak of the component. When the chromatogram is recorded from a gas-density balance, the air peak is an obvious positive deflection and the retention measurements are easily made. However, with the use of an ionization chamber detector the air peak is a negative deflection and is frequently not seen (Fig. 1). In this case, the time of the appearance of the carrier gas front can be calculated in the manner described below, and the retention times of all components can be measured from this "mathematical air peak."

By definition, the volume of gas (uncorrected for compressibility) required to elute a component after the appearance of the gas front is given as

$$V_R' = tF_c = \frac{xF_c}{S} \tag{1}$$

where t = retention time (minutes) which elapses from the appearance of the gas front to the appearance of the peak of a component, F_c = flow rate (cc. per min.) of the carrier gas measured at the outlet pressure and temperature of the column, x = distance (cm.) measured on the chart from the carrier gas front to the component peak and S = speed (cm. per min.) of the recorder chart.

From equation 1, the relationship between V_R' and the chain length,

$$\log V_R' \propto n \tag{2}$$

can be expressed

$$\log x \propto n \tag{3}$$

If the distance to a given peak is not measured from the true carrier gas front (x_0) but from some arbitrarily chosen point (y_0), this distance y will differ from x by δ, as shown in Figure 1. Thus,

$$x = y + \delta \tag{4}$$

In turn, equation 3 can be expressed

$$\log (y + \delta) \propto n \tag{5}$$

If three homologues in a chromatogram are chosen such that

$$n_2 - n_1 = n_3 - n_2 \tag{6}$$

then it follows from equation 5 that

$$\frac{y_2 + \delta}{y_1 + \delta} = \frac{y_3 + \delta}{y_2 + \delta} \tag{7}$$

or

$$\delta = \frac{{y_2}^2 - y_3 y_1}{y_3 + y_1 - 2y_2} \tag{8}$$

Accordingly, one can measure from any arbitrary point (y_0) to the peaks of three homologues which obey equation 6, record these distances as y_1, y_2, y_3 respectively, solve for δ and measure from this arbitrary point to the point which should be the location of the carrier gas front. If δ is positive, x_0 precedes y_0; if δ is negative, x_0 follows y_0.

A simplification of this calculation is to designate the peak of homologue 2 as y_0. In this case y_2 is zero. Then the distance between the peaks of homologues 2 and 1 multiplied by that between homologues 2 and 3, divided by their sum (y_1 is a negative number in this formulation), is the distance from the peak of homologue 2 to x_0.

In Figure 2 is shown the plot of the relative retentions, r (relative to methyl stearate), of the n-saturated components of the chromatogram shown in Figure 1. As measured from y_0 there is an obvious deflection from linearity, but when measured from x_0 these values show a strictly linear relationship with chain length.

As mentioned above, it has been customary to designate the air peak as the carrier gas front (1). However, when chromatographic analyses are made by the

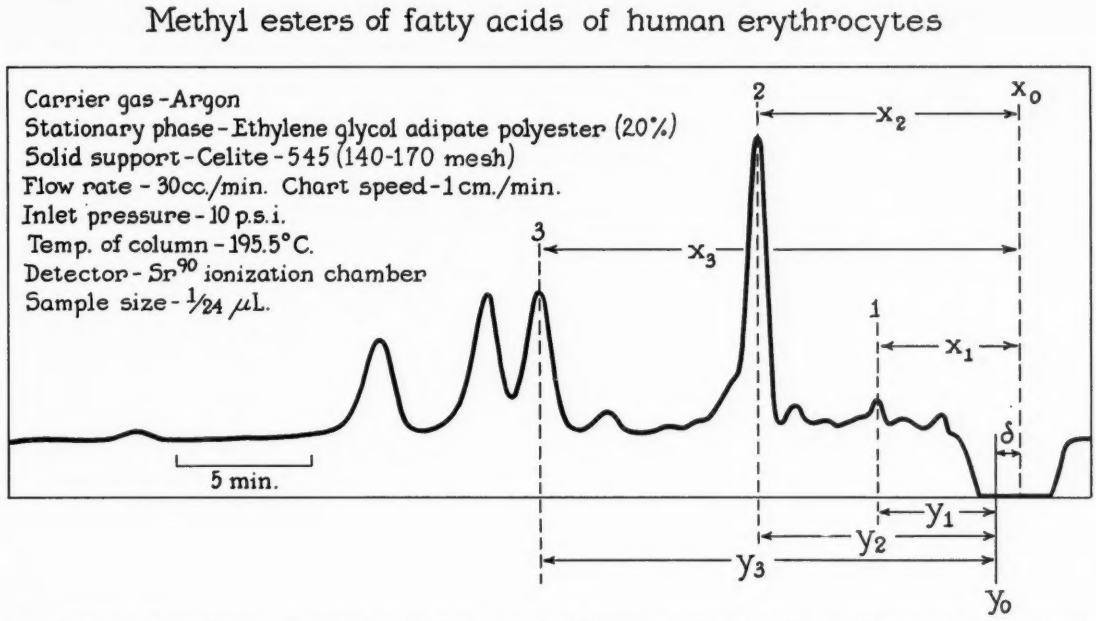

FIG. 1. Gas-liquid chromatogram of methyl esters of fatty acids of human erythrocytes. The homologues chosen for calculation of the location of the carrier gas front, x_0, are methyl myristate (1), methyl palmitate (2), and methyl stearate (3). The distances from an arbitrary point (y_0) to these homologues are designated y_1, y_2, y_3, respectively. For purposes of clarity the entire chromatogram is not shown, nor are all the components labeled. The chromatogram proceeds from right to left.

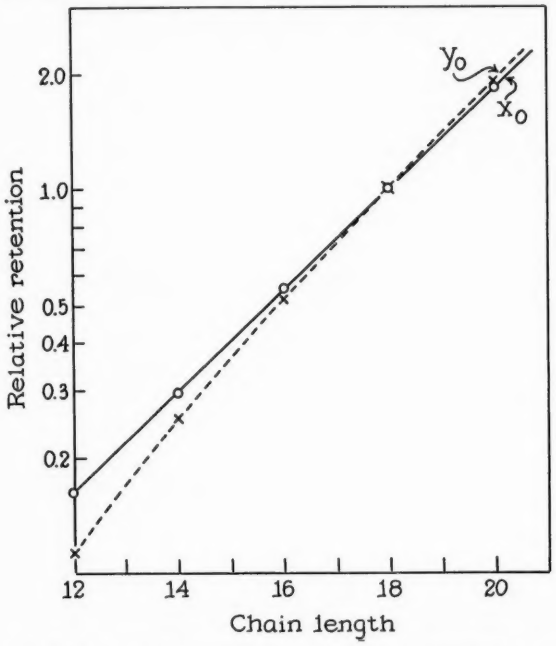

FIG. 2. Relative retentions of *n*-saturated components (relative to methyl stearate) as a function of the chain length. The broken line is the plot of the relative retentions of the components shown in Fig. 1, calculated from y_0. The solid line is the same plot for values calculated from x_0.

gas-flow interruption technique of sample loading, the carrier gas front calculated from equation 8 *precedes* the air deflection by a small but perceptible distance. This occurs in records made by the gas-density balance (Fig. 3) as well as by the ionization chamber detector (Fig. 4). This discrepancy is thought to be a function of the following considerations:

(*a*) The calculation presented above assumes a constant gradient between inlet and outlet pressures. This is not the case when the gas-flow interruption technique is used, since, when gas pressure is reapplied after loading the sample, the pressure gradient is not re-established instantaneously. Because the partition coefficient and hence the V_R' of every component is a function of gas pressure, any deviation from constancy of pressure invalidates the relationship expressed in equation 2. During this period of disequilibrium the components which partition most readily into the gas phase are subjected to the greatest distortion of V_R', whereas the components with the greater preference for stationary liquid phase (which appear last in the chromatographic run) are least affected. Figure 4 demonstrates that the calculated carrier gas front almost coincides with the air deflection when the calculation is made with three esters appearing late in the chromatographic analysis. How-

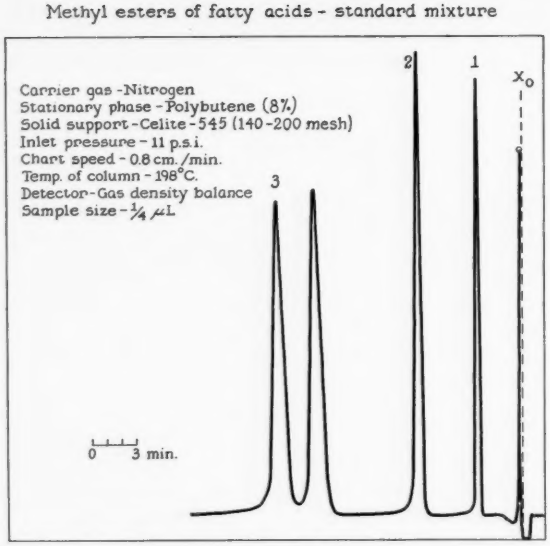
Methyl esters of fatty acids - standard mixture
Carrier gas - Nitrogen
Stationary phase - Polybutene (8%)
Solid support - Celite - 545 (140 - 200 mesh)
Inlet pressure - 11 p.s.i.
Chart speed - 0.8 cm./min.
Temp. of column - 198°C.
Detector - Gas density balance
Sample size - ¼ μL
3
2
1
x_0
0 3 min.

FIG. 3. Chromatographic analysis utilizing a gas-density balance. Methyl esters of myristic, palmitic, and stearic acids are designated *1*, *2*, and *3*, respectively. The unlabeled peak is methyl oleate. The calculated carrier gas front, x_0, precedes the air peak.

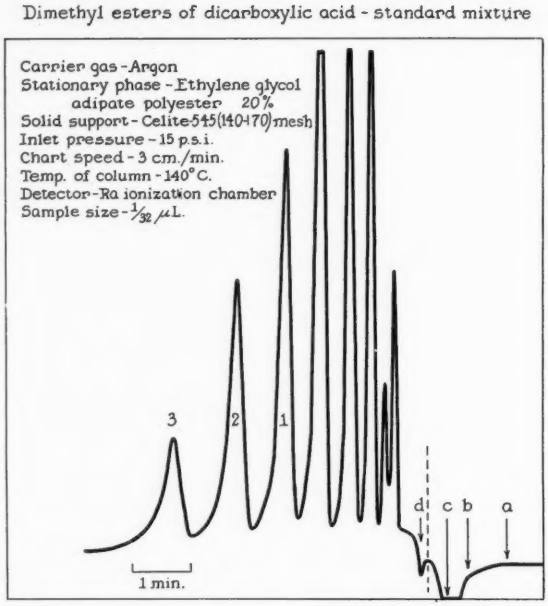

FIG. 4. Chromatographic analysis utilizing an ionization chamber detector. The gas-flow interruption technique of sample loading is used, illustrating the sequence of events at the start of the run. The carrier gas was obstructed (*a*), the inlet pressure fell to 5 pounds per square inch and the column was opened (*b*) to allow insertion of the sample. After the sample was applied, the carrier gas flow was re-established and the column closed (*c*). With this detection system the emergence of the air introduced with the sample appears as a negative deflection (*d*). The calculated carrier gas front (dotted line) precedes the air deflection. This calculation was made from the peaks designated *1*, *2*, and *3* (dimethyl esters of pimelic, suberic, and azelaic acids respectively).

ever, had the calculation been based on three early-appearing components, the discrepancy between the calculated carrier gas front and the air deflection would have been much larger. Thus the calculation of the location of the carrier gas front is most reliable when it is based on the three homologues with the longest retention times.

(*b*) The air deflection is created by components of the atmosphere which are introduced when the sample is placed on the column. If these distribute into the liquid phase, the air deflection is retarded.

When no air deflection is visible, the calculation of the location of the carrier gas front is made possible if familiar components are recognized which allow the application of equation 6. Then, from the retention volumes based on this calculated gas front, tentative identifications of unknown components can be made by reference to published tables of relative retentions.

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Volume 1
Number 1

New Methods

► **New Methods is a listing of recent and pertinent references to analytical lipid methods published in other journals. It is compiled by Norman S. Radin, H. A. Newman, and D. B. Zilversmit.**

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Scope: The JOURNAL OF LIPID RESEARCH, a quarterly, will publish original articles dealing with the chemistry, biochemistry, enzymology, histochemistry, and physiology of the lipids. The editors will favorably consider significant contributions in the field of lipid methodology and will encourage publication of sufficient details so that the methods can be reproduced. Clinical observations and nutritional data will be welcomed if they offer contributions in the above fields. In addition to original articles, the JOURNAL will contain review articles and a section, "Notes on Methodology," dealing primarily with minor improvements in existing methodology. Notes on Methodology will not generally require a summary or subdivision into sections. A listing of new lipid methods published in other journals will also be provided. Although articles will be published in English only, it is hoped that the JOURNAL will become a vehicle for international communication.

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kilogram	kg.
gram	g.
milligram	mg.
microgram	μg.
millimole	mmole (not mM)
micromole	μmole (not μM)
Units of Concentration:	
molar (mole per liter)	M
millimolar	mM
micromolar	μM
Units of Length, Area, Volume, etc.:	
meter	m.
centimeter	cm.
millimicron	mμ.
square centimeter	cm^2
liter	l.
milliliter	ml.
cubic centimeter	cc. or $cm.^3$
microliter	μl.
counts per minute	cpm.
millicurie	mc.
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